Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/043999

International filing date: 31 December 2004 (31.12.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/533,537
Filing date: 31 December 2003 (31.12.2003)

Date of receipt at the International Bureau: 09 February 2005 (09.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
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APPLICATION NUMBER: *60/533,537*
FILING DATE: *December 31, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/43999*

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Express Mail No.: EL 992621158 US
Attorney Docket No.: 2004367-0027
Date Filed: December 31, 2003

PROVISIONAL APPLICATION TRANSMITTAL

(REQUEST FOR FILING A PROVISIONAL APPLICATION FOR PATENT UNDER 37 CFR § 1.53(C))

Dear Sir:

Please find enclosed a provisional patent application and papers as follows for:

Inventor(s):

Given Name (first and middle)	Family Name or Surname	Residence (City and State or Foreign Country)
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Title of the Invention: IMPROVED BONE GRAFT METHODS AND COMPOSITIONS

A) ENCLOSED APPLICATION PARTS:

1) Comprising: _88_ pages of specification (including 1 page of abstract and 23 pages of claims) and 1 page of drawing **(Total 89 pages)**;

B) OTHER ACCOMPANYING APPLICATION PARTS:

2) _X_ Return Receipt Postcard (MPEP § 503) (specifically itemized)

3) ___ Application Data Sheet. See 37 CFR § 1.76

4) ___OTHER: (if applicable, specified below)

C) CORRESPONDENCE ADDRESS:

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D) METHOD OF PAYMENT OF FILING FEES:

_X_ Applicant claims small entity status. See 37 CFR §1.27.

___ Statement Verifying Small Entity Status (optional)

_X_ A check or money order is enclosed to cover the filing fees.

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FILING FEE AMOUNT ($): $ **80.00**

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__X__ NO.

_____ YES, THE NAME OF THE U.S. GOVERNMENT AGENCY AND THE GOVERNMENT CONTRACT NUMBER ARE : .

Respectfully Submitted,

Dated: **December 31, 2003**

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IMPROVED BONE GRAFT METHODS AND COMPOSITIONS

Background

[01] The rapid and effective repair of bone and cartilage defects caused by injury, disease, wounds, surgery, *etc.*, has long been a goal of orthopaedic surgery. Toward this end, a number of compositions and materials have been used or proposed for use in the repair of bone and cartilage defects. The biological, physical, and mechanical properties of the compositions and materials are among the major factors influencing their suitability and performance in various orthopaedic applications.

[02] Autologous cancellous bone ("ACB") is considered the gold standard for bone grafts. ACB is osteoinductive, is non-immunogenic, and, by definition, has all of the appropriate structural and functional characteristics appropriate for the particular recipient. Unfortunately, ACB is only available in a limited number of circumstances. Some individuals lack ACB of appropriate dimensions and quality for transplantation. Moreover, donor site morbidity can pose serious problems for patients and their physicians.

[03] Much effort has been invested in the identification or development of alternative bone graft materials. Demineralized bone matrix ("DBM") implants have been reported to be particularly useful (see, for example, U.S. Patents 4,394,370; 4,440,750; 4,485,097; 4,678,470; and 4,743,259; Mulliken et al., *Calcif. Tissue Int.* 33:71, 1981; Neigel *et al., Opthal. Plast. Reconstr. Surg.* 12:108, 1996; Whiteman et al., *J. Hand. Surg.* 18B:487, 1993; Xiaobo et al., *Clin. Orthop.* 293:360, 1993; each of which is incorporated herein by reference). Demineralized bone matrix is typically derived from cadavers. The bone is removed aseptically and/or treated to kill any infectious agents. The bone is then particulated by milling or grinding and then the mineral component is extracted (*e.g.*, by soaking the bone in an acidic solution). The remaining matrix is malleable and can be further processed and/or formed and shaped for implantation into a particular site in the recipient. Demineralized bone prepared in this manner contains a variety of components including proteins, glycoproteins, growth factors, and proteoglycans. Following implantation, the presence of DBM induces cellular recruitment to the site of injury. The recruited cells may eventually differentiate into bone forming cells. Such recruitment of cells leads to an increase in the rate of wound healing and, therefore, to faster recovery for the patient.

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[04] Current methods of articular cartilage restoration include (1) stimulation of fibrocartilaginous repair; (2) osteochondral grafting; and (3) autologous chondrocyte implantation. The results achieved using fibrocartilagenous repair are difficult to assess and deteriorate over time. Osteochondral grafting requires harvesting of cartilage with a layer of subchondral bone and implanting it into the articular defect site. The graft is fixed to the host by healing onto the host bone. Osteochondral grafts have the mechanical properties of normal articular cartilage, but this technique risks donor site morbidity and disease transmission.

[05] Autologous chondrocyte implantation introduces isolated chondrocytes into the defect site after a period of *ex vivo* processing (see, e.g., U.S. Patent Nos. 5,041,138; 5,206,023; 5,786,217; and 6,080,194, incorporated herein by reference). The cells are contained *in vivo* by a patch of periosteum, which is sutured to the surrounding host cartilage. The cells attach to the defect walls and produce extracellular matrix *in situ*. Although being able to use autologous cells and expand the cells *ex vivo* are significant advantages of this technique, loss of cell adherence, phenotypic dedifferentiation, and extracellular matrix production are proven difficulties.

[06] A variety of approaches have been explored in an attempt to recruit progenitor cells or chondrocytes into an osteochondral or chondral defect. For example, penetration of subchondral bone has been performed in order to access mesenchymal stem cells (MSCs) in the bone marrow, which have the potential to differentiate into cartilage and bone. (Steadman, et al., "Microfracture: Surgical Technique and Rehabilitation to Treat Chondral Defects", *Clin. Orthop.*, 391 S:362-369 (2001). In addition, some factors in the body are believed to aid in the repair of cartilage. For example, it has been observed that transforming growth factors beta (TGF-β) have the capacity to recruit progenitor cells into a chondral defect from the synovium or elsewhere when TGF-β is loaded in the defect (Hunziker, et al., "Repair of Partial-Thickness Defects in Articular Cartilage: Cell Recruitment From the Synovial Membrane", *J. Bone Joint Surg.*, 78-A:721-733 (1996)). However, the application of growth factors to bone and cartilage implants has not resulted in the increase in osteoinductive or chondrogenic activity, respectively, expected.

[07] Each of U.S. Patent Nos. 5,270,300 and 5,041,138 describes a method for treating defects or lesions in cartilage which provides a matrix, possibly composed of collagen, with pores,

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which are large enough to allow cell population and contain growth factors (e.g., TGF-β) or other factors (e.g. angiogenesis factors) appropriate for the type of tissue desired to be regenerated.

[08] Overall, current bone and cartilage graft formulations have various drawbacks. First, while the structures of most bone or cartilage matrices are relatively stable, the active factors within the matrices are rapidly degraded. The osteogenic or chondrogenic activity of the bone or cartilage matrix may be significantly degraded within 24 hours after implantation, and in some instances the osteogenic or chondrogenic activity may be inactivated within 6 hours. Therefore, the factors associated with the matrix are only available to recruit cells to the site of injury for a short time after transplantation. For much of the healing process, which may take weeks to months, the implanted material may provide little or no assistance in recruiting cells.

Summary of the Invention

[09] The present invention provides improved bone and cartilage matrices and methods for their production. In preferred embodiments of the invention the matrices contain peptides or protein fragments that increase the osteoinductive or chondrogenic properties of the bone or cartilage matrices, respectively. The peptides or protein fragments may be exogenously added to the matrix. Alternately, or in addition to this approach, in certain embodiments of the invention the matrix is treated, e.g., with a protease, chemical, or condition, that cleaves proteins present in the matrix (e.g., proteins such as bone morphogenetic proteins), resulting in conversion of an inactive protein into an active form, and/or generating an active molecule that is less susceptible to degradation than a longer molecule from which it is derived. The invention also encompasses matrices comprising other agents, e.g., agents that improve the osteogenic and/or chondrogenic activity of the matrix by either transcriptional or post-transcriptional regulation of the synthesis of bone or cartilage enhancing or inhibiting factors by cells within the matrix.

[10] In a first aspect, the invention provides a method of increasing the osteoinductivity of a bone matrix, the method comprising the steps of: (i) providing a bone matrix; and (ii) contacting the bone matrix with at least one protease that cleaves at least one protein to generate osteoinductive peptides or protein fragments, wherein the peptides or protein fragments cause an increase in osteoinductivity of the bone matrix contacted with the protease resulting in improved bone formation compared to a bone matrix not contacted with the protease. The invention

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further provides a method of increasing the osteoinductivity of a bone matrix, the method comprising the steps of: (i) providing a bone matrix; and (ii) contacting the bone matrix with a chemical or condition that generates active osteoinductive peptides or protein fragments, wherein the peptides or protein fragments cause an increase in osteoinductivity of the bone matrix contacted with the protease resulting in improved bone formation compared to a bone matrix not contacted with the protease. In various embodiments of the invention the bone matrix comprises mineralized bone matrix, partially demineralized bone matrix, demineralized bone matrix, deorganified bone matrix, anorganic bone matrix, or a mixture thereof. In various embodiments of the invention the peptides or protein fragments are derived from a growth factor. The invention also provides a method of increasing the osteoinductivity of a bone matrix comprising the step of contacting a bone matrix with at least one protease that selectively degrades inhibitors of osteogenic activity, wherein the bone matrix has increased osteogenic activity resulting in improved bone formation compared to a bone matrix not contacted with the protease. Similar methods are provided for cartilage matrices.

[11] In another aspect, the invention provides an osteoinductive bone matrix composition for implantation at a bone defect site which comprises a bone matrix treated with at least one protease, wherein the protease causes cleavage of inactive proteins to generate osteoinductive peptides or protein fragments, wherein the osteoinductivity of the treated matrix compared to an untreated matrix is increased resulting in improved bone formation. In addition, the invention provides an osteoinductive bone matrix composition for implantation at a bone defect site which comprises a bone matrix treated with a chemical or condition, wherein the chemical or condition generates active osteoinductive peptides or protein fragments, wherein the osteoinductivity of the treated matrix compared to an untreated matrix is increased resulting in improved bone formation. In various embodiments of the invention the bone matrix comprises mineralized bone matrix, partially demineralized bone matrix, demineralized bone matrix, deorganified bone matrix, anorganic bone matrix, or a mixture thereof. In various embodiments of the invention the peptides or protein fragments are derived from a growth factor.

[12] In another aspect, the invention features a chondrogenic cartilage repair composition for implantation at a cartilage defect site which comprises a cartilage repair matrix treated with at least one protease, wherein the protease causes cleavage of inactive proteins to generate active chondrogenic peptides or protein fragments, wherein the chondrogenic activity of the treated

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matrix compared to an untreated matrix is increased resulting in improved cartilage formation. The invention further includes a chondrogenic cartilage repair composition for implantation at a cartilage defect site which comprises a cartilage repair matrix treated with a chemical or condition, wherein the chemical or condition generates active chondrogenic peptides or protein fragments, wherein the chondrogenic activity of the treated matrix compared to an untreated matrix is increased resulting in improved cartilage formation.

[13] In another aspect, the invention provides an implantable bone growth inducing composition comprising: (i) a bone matrix; and (ii) a peptide or protein fragment that is capable of enhancing the osteoinductivity of the bone matrix resulting in improved bone formation ability as compared to a composition without the peptide or protein fragment. The invention further features an implantable cartilage repair graft composition comprising: (i) a cartilage repair matrix; and (ii) at least one peptide or protein fragment that is capable of enhancing the chondrogenic activity of the cartilage repair matrix resulting in improved cartilage formation ability as compared to a composition without the peptide or protein fragment. The bone matrix component of the inventive compositions may comprise mineralized bone matrix, partially demineralized bone matrix, demineralized bone matrix, deorganified bone matrix, anorganic bone matrix, or mixtures thereof. In certain embodiments of the invention the peptide or protein fragment is derived from a growth factor.

[14] The invention further provides a method of preparing a bone matrix composition, the method comprising the steps of: (i) providing a bone matrix; and (ii) adsorbing into the bone matrix peptides or protein fragments that are capable of enhancing the osteoinductivity of the bone matrix resulting in improved bone formation ability as compared to a composition without the peptides or protein fragments. The invention also includes a method of preparing a cartilage repair matrix composition, the method comprising the steps of: (i) providing a cartilage repair matrix; and (ii) adsorbing into the matrix peptides or protein fragments that are capable of enhancing the chondrogenic activity of the cartilage repair matrix resulting in improved cartilage formation ability as compared to a composition without the peptides or protein fragments.

[15] In another aspect, the invention provides a bone or cartilage matrix composition comprising: (i) a bone or cartilage matrix; and (ii) an antisense nucleic acid that decreases expression of BCEF or BCIF mRNA, a ribozyme that cleaves a BCEF or BCIF mRNA, an RNAi-mediating agent that decreases expression of a BCEF or BCIF, or a vector that provides a

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template for transcription of any of the foregoing RNA molecules. The invention further provides a method of increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising the step of introducing an antisense nucleic acid that decreases expression of BCEF or BCIF mRNA, a ribozyme that cleaves a BCEF or BCIF mRNA, an RNAi-mediating agent that decreases expression of a BCEF or BCIF, or a vector that provides a template for transcription of any of the foregoing RNA molecules into the matrix.

[16] The invention further provides a bone or cartilage matrix composition comprising: (i) a bone or cartilage matrix; and (ii) a transcription modulator, wherein the transcription modulator modulates transcription of a BCEF or BCIF. The transcription modulator can be, for example, a small molecule, a transcription factor, an engineered transcription modulating protein, or a vector that provides a template for intracellular synthesis of a transcription factor or engineered transcription modulating protein. The invention also includes a method of increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising the step of introducing a transcription modulator into the matrix, wherein the transcription modulator modulates transcription of a BCEF or BCIF.

[17] The invention further provides methods of treating a bone or cartilage defect, or a disease or condition that results in deterioration of bone or cartilage, by implanting any of the various compositions of the invention into a subject.

[18] This application refers to various patents, patent applications, journal articles, and other publications, all of which are incorporated herein by reference. In addition, the following standard reference works are incorporated herein by reference: *Current Protocols in Molecular Biology*, *Current Protocols in Immunology, Current Protocols in Protein Science,* and *Current Protocols in Cell Biology*, John Wiley & Sons, N.Y., edition as of July 2002; Sambrook, Russell, and Sambrook, *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2001; Rodd 1989 "Chemistry of Carbon Compounds", vols. 1-5 and supps, Elsevier Science Publishers, 1989; "Organic Reactions", vols 1-40, John Wiley and Sons, New York, NY, 1991; March 2001, "Advanced Organic Chemistry", 5th ed. John Wiley and Sons, New York, NY.

Brief Description of the Drawing

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[19] Figure 1 illustrates how bone and/or cartilage preparations may be combined with enzymatic or chemical agents that act on their substrates to generate peptide(s) or proteins fragment(s) that enhance the osteogenic, and/or chondrogenic activity of the preparation(s).

Definitions

[20] *Anorganic, as* herein applied to matrices, particles, etc., refers to matrices or particles that were subjected to a process that removes their entire original organic content.

[21] A peptide or protein fragment, or a bioactive agent, is *associated with* a bone or cartilage matrix or material (e.g., a bone particle) or other osteoinductive or chondrogenic matrix or material according to the present invention if it is retained by the implant long enough to affect the osteoinductive or chondrogenic activity of the implant. In some embodiments, associations are covalent; in others they are non-covalent. The bioactive agent may be rendered *associated with* a matrix by virtue of a physical interaction with one or more entities that are themselves associated with the matrix. Various stabilizing agents that can cause association with matrix are described in U.S.S.N. 10/271,140, filed October 15, 2002, incorporated herein by reference.

[22] *Autograft*, is used herein to refer to a tissue that is extracted from the intended recipient of an implant. Such material will be considered to be an autograft, even if it is prepared, processed, and/or expanded in tissue culture.

[23] As used herein, the term *bioactive agent* or *bioactive compound* is used to refer to a compound or entity that alters, inhibits, activates, or otherwise affects biological or chemical events. For example, bioactive agents may include, but are not limited to, anti-AIDS substances, anti-cancer substances, antibiotics, immunosuppressants, anti-viral substances, enzyme inhibitors, hormones, neurotoxins, opioids, hypnotics, anti-histamines, lubricants, tranquilizers, anti-convulsants, muscle relaxants and anti-Parkinson substances, anti-spasmodics and muscle contractants including channel blockers, miotics and anti-cholinergics, anti-glaucoma compounds, anti-parasite and/or anti-protozoal compounds, modulators of cell-extracellular matrix interactions including cell growth inhibitors and anti-adhesion molecules, vasodilating agents, inhibitors of DNA, RNA or protein synthesis, anti-hypertensives, analgesics, anti-pyretics, steroidal and non-steroidal anti-inflammatory agents, anti-angiogenic factors, anti-

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secretory factors, anticoagulants and/or antithrombotic agents, local anesthetics, ophthalmics, prostaglandins, anti-depressants, anti-psychotic substances, anti-emetics, and imaging agents. In a certain preferred embodiments, the bioactive agent is a drug.

[24] A more complete listing of bioactive agents and specific drugs suitable for use in the present invention may be found in "Pharmaceutical Substances: Syntheses, Patents, Applications" by Axel Kleemann and Jurgen Engel, Thieme Medical Publishing, 1999; the "Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals", Edited by Susan Budavari *et al.*, CRC Press, 1996; and the United States Pharmacopeia-25/National Formulary-20, published by the United States Pharmcopeial Convention, Inc., Rockville MD, 2001, each of which is incorporated herein by reference.

[25] The term *biocompatible*, as used herein, is intended to describe materials that upon administration *in vivo*, do not induce undesirable long-term effects.

[26] A *bone growth factor*, as used herein, is any compound or agent that enhances or stimulates bone growth and/or repair.

[27] A *cartilage growth factor*, as used herein, is any compound or agent that enhances or stimulates cartilage growth and/or repair.

[28] *Chemotactic*, as used herein, means a substance having the ability to recruit cells from the host that have the potential for forming or repairing new bone or cartilage tissue and/or for contributing to such formation or repair (e.g., by providing growth factors). Certain chemotactic agents may also function as proliferation agents.

[29] *Chondrogenic*, as used herein, means giving rise to or forming cartilage.

[30] *Chondrogenic activity* refers to the cartilage forming ability of a cartilage repair matrix.

[31] *Demineralized bone activity* refers to the osteoinductive activity of demineralized bone.

[32] *Demineralized*, as used herein (e.g., in reference to a matrix), refers to any material generated by removing mineral material from living tissue, e.g., bone tissue. In preferred embodiments, the demineralized compositions described herein include preparations containing less than 5% calcium and preferably less than 1% calcium by weight. Partially demineralized bone (*e.g.*, preparations with greater than 5% calcium by weight but containing less than 100% of the original starting amount of calcium) is also considered within the scope of the invention. In general, demineralized bone has less than 95% of its original mineral content.

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[33] *Deorganified,* as herein applied to matrices, particles, etc., refers to bone or cartilage matrices, particles, etc., that were subjected to a process that removes part of their original organic content.

[34] *Generates*, as used herein, means to yield or to result in release of peptides and protein fragments. For example, a protease, chemical, or condition of the present invention can be contacted with a bone matrix to generate peptides and protein fragments having osteoinductive capability. The peptides can be generated, for example, by cleavage of a protein into active peptides or protein fragments, dissociation from a cofactor, changing the conformation of a peptide or protein, *etc.*

[35] *Matrix*, as used herein, refers to a natural or non-natural substantially solid vehicle capable of association with at least one peptide or protein fragment for delivery to an implant site, for example, active peptides or protein fragments derived from growth factors such as bone morphogenic proteins or transforming growth factors. The matrix may be completely insoluble or may be slowly solubilized after implantation. Following implantation, preferred matrices resorb or degrade, remaining substantially intact for at least one to seven days, most preferably for two or four weeks or longer and often longer than 60 days. Bioactive agents may be endogenously present in the matrix as in the case of most demineralized bone, or they may be exogenously added to the matrix. Matrices may also comprise combinations of endogenous and exogenous bioactive agents. The matrix may be in particulate or fiber form, or may be monolithic. The matrix may comprise a number of materials and forms in combination such as fibers and particles (see, e.g., U.S.S.N. 10/271,140, filed October 15, 2002, incorporated herein by reference). The most preferred matrices are calcium phosphates, the preparation of which is well known to practitioners in the art (see, for example, Driessens *et al.* "Calcium phosphate bone cements" Wise, D. L., Ed. *Encyclopedic Handbook of Biomaterials and Bioengineering, Part B, Applications* New York: Marcel Decker; Elliott *Structure and Chemistry of the Apatites and Other Calcium Phosphates* Elsevier, Amsterdam, 1994; each of which is incorporated herein by reference). Calcium phosphate matrices include, but are not limited to, dicalcium phosphate dihydrate, monetite, tricalcium phospate, tetracalcium phosphate, hydroxyapatite, nanocrystalline hydroxyapatite, poorly crystalline hydroxyapatite, substituted hydroxyapatite, and calcium deficient hydroxyapatites.

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[36] As used herein, *operably linked* or *operably associated* refers to a relationship between two nucleic acid sequences wherein the expression of one of the nucleic acid sequences is controlled by, regulated by, modulated by, etc., the other nucleic acid sequence. For example, the transcription of a nucleic acid sequence is directed by an operably linked promoter sequence; post-transcriptional processing of a nucleic acid is directed by an operably linked processing sequence; the translation of a nucleic acid sequence is directed by an operably linked translational regulatory sequence; the transport or localization of a nucleic acid or polypeptide is directed by an operably linked transport or localization sequence; and the post-translational processing of a polypeptide is directed by an operably linked processing sequence. Preferably a nucleic acid sequence that is operably linked to a second nucleic acid sequence is covalently linked, either directly or indirectly, to such a sequence, although any effective three-dimensional association is acceptable.

[37] *Osteogenic* is used herein to refer to the ability of an implant to enhance or accelerate the growth of new bone tissue by one or more mechanisms such as osteogenesis, osteoconduction, and/or osteoinduction.

[38] *Osteoinductive*, as used herein, refers to the quality of being able to recruit cells from the host that have the potential to stimulate new bone formation. Any material that can induce the formation of ectopic bone in the soft tissue of an animal is considered osteoinductive. For example, most osteoinductive materials induce bone formation in athymic rats when assayed according to the method of Edwards et al. ("Osteoinduction of Human Demineralized Bone: Characterization in a Rat Model" *Clinical Orthopeadics& Rel. Res.*, 357:219-228, December 1998; incorporated herein by reference). In other instances, osteoinduction is considered to occur through cellular recruitment and induction of the recruited cells to an osteogenic phenotype. Osteoinductivity may also be determined in tissue culture as the ability to induce an osteogenic phenotype in culture cells (primary, secondary, or explants). It is advisable to calibrate the tissue culture method with an *in vivo* ectopic bone formation assay as described by Zhang et al. ("A quantitative assessment of osteoinductivity of human demineralized bone matrix" *J. Periodontol.* 68(11):1076-84, November 1997; incorporated herein by reference). Calibration of the *in vitro* assays against an art-accepted *in vivo* ectopic bone formation model is important because the ability of a compound to induce an apparent "osteogenic" phenotype in tissue culture may not always be correlated with the induction of new bone formation *in vivo*.

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BMP, IGF, TGF-β, and angiogenic factors are among the osteoinductive factors found to recruit cells from the marrow or perivascular space to the site of injury and then cause the differentiation of these recruited cells down a pathway responsible for bone formation. DBM isolated from either bone or dentin are both osteoinductive materials (Ray et al., "Bone implants" *J. Bone Joint Surgery* 39A:1119, 1957; Urist, "Bone: formation by autoinduction" *Science* 150:893, 1965; each of which is incorporated herein by reference).

[39] *Osteoinductivity score* refers to a score ranging from 0 to 4 as determined according to the method of Edwards et al. (1998) or an equivalent calibrated test. In the method of Edwards et al., a score of "0" represents no new bone formation; "1" represents 1%-25% of implant involved in new bone formation; "2" represents 26-50% of implant involved in new bone formation; "3" represents 51%-75% of implant involved in new bone formation; and "4" represents >75% of implant involved in new bone formation. In most instances, the score is assessed 28 days after implantation. However, the osteoinductive score may be obtained at earlier time points such as 7, 14, or 21 days following implantation. In these instances it is important to include a normal DBM control such as DBM powder without a carrier, and if possible, a positive control such as BMP. Occasionally osteoinductivity may also be scored at later timepoints such as 40, 60, or even 100 days following implantation. Percentage of osteoinductivity refers to an osteoinductivity score at a given time point expressed as a percentage of activity, of a specified reference score.

[40] *Osteoconductive*, is used herein to refer to the ability of a non-osteoinductive substance to serve as a suitable template or substance along which bone may grow.

[41] *Proteases*, as used herein, are protein-cleaving enzymes that cleave peptide bonds that link amino acids in protein molecules to generate peptides and protein fragments. A large collection of proteases and protease families has been identified. Some exemplary proteases include serine proteases, aspartyl proteases, acid proteases, alkaline proteases, metalloproteases, carboxypeptidase, aminopeptidase, cysteine protease, *etc.* A particularly preferred family of proteases is the proprotein convertase family, which includes furin (Dubois et al., *American Journal of Pathology* (2001) 158(1):305-316). Members of the proprotein convertase family of proteases are known to proteolytically process proTGFs and proBMPs to their active mature forms (Dubois et al., *American Journal of Pathology* (2001) 158(1):305-316; Cui et al., *The Embo Journal* (1998) 17(16):4735-4743; Cui et al., *Genes & Development* (2001) 15:2797-2802,

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each incorporated by reference herein). Preferred proteases are protein or peptide based. Certain preferred proteases are commercially available from chemical companies such as Aldrich-Sigma.

[42] A *peptide* or *protein fragment*, according to the present invention, comprises a string of at least two amino acids linked together by peptide bond(s). Inventive peptides and protein fragments preferably contain only natural amino acids, although non-natural amino acids (*i.e.*, compounds that do not occur in nature but that can be incorporated into a polypeptide chain) and/or amino acid analogs as are known in the art may alternatively be employed. Also, one or more of the amino acids in an inventive peptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, *etc.* In various embodiments of the invention peptides and protein fragments may be cleavage products of longer proteins, e.g., proproteins, biologically inactive longer proteins, biologically active longer proteins, etc., which may or may not have undergone one or more posttranslational processing events.

[43] *Proliferation agent* and *mitogenic agent* are used herein interchangeably to refer to the ability of a substance to enhance the proliferation of cells from the host that have the potential to form new bone or cartilage or repair new bone or cartilage.

[44] *Purified*, as used herein referring to the purification of a protein, means to be separated from some or all of the components with which a protein is normally associated in a host cell. The host cell can be a host cell that expresses the protein naturally (i.e., in nature, or a genetically modified host cell that expresses the protein as a result of genetic engineering.

[45] As used herein, the term *small molecule* refers to organic compounds, whether naturally-occurring or artificially created (*e.g.*, via chemical synthesis) that have relatively low molecular weight and that are not proteins, polypeptides, or nucleic acids. Typically, small molecules have a molecular weight of less than about 1500 g/mol. Also, small molecules typically have multiple carbon-carbon bonds.

[46] The term *subject,* as used herein, refers to an individual to whom an agent such as a bone repair matrix or cartilage repair matrix of the invention is to be delivered, e.g., for experimental, diagnostic, and/or therapeutic purposes. Preferred subjects are mammals, particularly domesticated mammals (e.g., dogs, cats, etc.), primates, or humans.

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[47] *Targeting agent* is any chemical entity that, when included in an inventive composition, will direct the composition to a particular site or cause the inventive composition to remain in a particular site within the recipient's body. A targeting agent may be a small molecule, peptide, protein, biological molecule, polynucleotide, *etc.* Typical targeting agents are antibodies, ligands of known receptors, and receptors. These targeting agents may be associated with the inventive composition through covalent or non-covalent interactions so that the inventive composition is directed to a particular tissue, organ, injured site, or cell type. A targeting agent , for example, may be associated with a peptide or protein fragment having osteoinductive or chondrogenic activity.

[48] A *transforming agent*, as used herein, is a substance that induces the development or change of any repair cells from the host into bone or cartilage forming cells, e.g., osteocytes or chondrocytes. Preferably these cells are capable of producing the factors typical for an osteocyte or chondroctye. For example, the chondrocytes are capable of producing cartilage-specific proteoglycans and type II collagen.

[49] In general, the term *vector* refers to a nucleic acid molecule capable of mediating entry of, e.g., transferring, transporting, etc., a second nucleic acid molecule into a cell. The transferred nucleic acid is generally linked to, e.g., inserted into, the vector nucleic acid molecule. A vector may include sequences that direct autonomous replication, or may include sequences sufficient to allow integration into host cell DNA. Useful vectors include, for example, plasmids (typically DNA molecules although RNA plasmids are also known), cosmids, and viral vectors. As is well known in the art, the term *viral vector* may refer either to a nucleic acid molecule (e.g., a plasmid) that includes virus-derived nucleic acid elements that typically facilitate transfer or integration of the nucleic acid molecule (examples include retroviral or lentiviral vectors) or to a virus or viral particle that mediates nucleic acid transfer (examples include retroviruses or lentiviruses). As will be evident to one of ordinary skill in the art, viral vectors may include various viral components in addition to nucleic acid(s).

[50] *Xenogenic* or *xenogeneic* is used herein to refer to a material intended for implantation that is obtained from a donor source of a different species than the intended recipient. For example, when the implant is intended for use in an animal such as a horse (equine), xenogenic tissue of, for example, bovine, porcine, caprine, etc, origin may be suitable.

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Detailed Description of Certain Embodiments

I. Introduction

[51] The present invention provides compositions and methods relating to improved bone and cartilage matrices, which contain peptides or protein fragments that increase the osteoinductive or chondrogenic properties of the bone or cartilage matrices, respectively. Below, certain aspects of preferred embodiments of the invention are described in more detail. Those of ordinary skill will appreciate that a variety of embodiments or versions of the invention are not specifically discussed below but are nonetheless within the scope of the present invention, as defined by the appended claims.

[52] Bone is made up of collagen, mineral, and other non-collagenous proteins. The mineral, primarily hydroxyapatite, is made of very fine, poorly formed crystals, with a large surface area in some bone, such as cancellous bone, that is reasonably capable of resorption and remodeling. Cortical bone, on the other hand, has a compound structure comprised of calcium hydroxyapatite reinforced with collagen fiber. Because it has greater mechanical strength, cortical bone is positioned to receive the greatest physical loads generated by the weight of the body and skeletal muscle contractions. Cancellous bone composes the "head" (epiphysis) and the inner areas of the bones, and borders the medullary cavity in the larger bones.

[53] Bone matrices can be mineralized, partially demineralized, demineralized, deorganified, anorganic, or mixtures of mineralized, partially demineralized, demineralized, deorganified, and/or anorganic. The present invention utilizes any one or a combination of mineralized, partially demineralized, demineralized, deorganified, or anorganic bone matrix. Demineralized bone matrix (DBM), as described herein, is comprised principally of proteins and glycoproteins, collagen being the primary protein substituent of DBM. While collagen is relatively stable, being degraded only by the relatively rare collagenase enzymes, various other proteins and active factors present in DBM are quickly degraded by enzymes present in the host. These host-derived enzymes include proteases and sugar-degrading enzymes (*e.g.*, endo- and exo-glycosidases, glycanases, glycolases, amylase, pectinases, galacatosidases, *etc.*). Thus growth factor proteins in a DBM or added to a DBM may have a limited osteoinductive effect because they are rapidly inactivated by the proteolytic environment of the DBM.

[54] A similar problem arises in cartilage matrices, which also rely on growth factors and other proteins that have a chondrogenic function or attract cells having a chondrogenic function.

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Cartilage is an avascular tissue composed of 5-10% by weight of living cells. There are three major types of cartilage in the body: hyaline (or articular) cartilage, fibrocartilage, and elastic cartilage. Articular cartilage covers the epiphyses of the bone and, in synovial joints, lies within a fluid-filled capsule. Articular cartilage is load-bearing tissue that distributes forces across joint surfaces, protects the more rigid underlying bone, and provides smooth articulation and bending of the joints during normal activities of daily living. Fibrocartilage composes the intervertebral discs separating the vertebrae of the spinal columns. Elastic cartilage is present in areas requiring extreme resilience, such as the tip of the nose.

[55] Certain cartilage matrices are described in U.S. Patent Nos. 5,270,300 and 5,041,138, incorporated herein by reference. Each of these patents describes a method for treating defects or lesions in cartilage, which provides a matrix, possibly composed of collagen, with pores large enough to allow cell population. The matrices further contain growth factors or other factors (e.g. angiogenesis factors) appropriate for the type of tissue regenerated. For example, TGF-β is added to the matrix as a proliferation and chemotactic agent to induce differentiation of cartilage repair cells. However, such factors are frequently inactivated once they are implanted, resulting in a reduction in chondrogenic activity of cartilage matrices over time. Additional matrices for the generation and/or repair of cartilage include matrices comprising hydrogels, polymers, etc.

II. Increasing the Activity of a Bone or Cartilage Matrix Using Peptides, Peptide Fragments, and Proteases

[56] The present invention provides methods for increasing the osteogenic or chondrogenic activity of a bone or cartilage matrix, respectively, as well as bone or cartilage matrix compositions that generate peptides and protein fragments having osteoinductivity or chondrogenic activity, respectively. In contrast to various longer proteins, certain peptides and protein fragments are less susceptible to proteolytic degradation and more likely to maintain their osteoinductive or chondrogenic properties in the proteolytic environment of the matrix. Many osteoinductive and chondrogenic proteins, for example, growth factors such as bone morphogenic protein (BMP), cell signaling molecules, transcription factors, hormones, *etc.*, have domains that are responsible for binding to receptors and initiating signal transduction in bone and cartilage growth pathways. These domains are capable of functioning independently as peptides and protein fragments. In certain embodiments, the present invention increases the

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osteoinductive or chondrogenic activity of bone and cartilage matrices by cleaving the osteoinductive and chondrogenic factors present in the matrix to generate active peptides or protein fragments and/or to generate active peptides or protein fragments that are less susceptible to degradation than their longer precursors. The increased number of factors in the matrix results in increased bone or cartilage formation.

[57] In one preferred embodiment, the present invention provides methods of increasing the osteoinductivity of a bone matrix. As shown in Figure 1 (left side) ccording to certain embodiments of the present invention, a bone matrix composition, either mineralized, partially demineralized, demineralized, deorganified, anorganic, or a combination thereof, is contacted with at least one enzyme, such as a protease that cleaves one or more proteins in the bone matrix to generate peptides or protein fragments having osteoinductive activity. Without limiting the theory of the present invention, the peptides or protein fragments generated, because they are already broken down and are less susceptible to further proteolytic degradation relative to the longer proteins from which they were derived, cause an increase in osteoinductivity of the bone matrix compared to a bone matrix not treated with a protease. The increase is also persistent over time since the peptides or protein fragments outlast longer protein precursors, which are subject to proteolytic breakdown.

[58] In other preferred embodiments, the present invention provides methods of increasing the chondrogenic activity of a cartilage repair matrix by providing a cartilage matrix and contacting a cartilage repair matrix with at least one protease that cleaves one or more proteins to generate peptides or protein fragments having chondrogenic activity. Since the peptides and protein fragments are not readily enzymatically degraded, generation of the active domains causes an increase in chondrogenic activity in the cartilage repair matrix compared to a cartilage repair matrix lacking a protease.

[59] In addition to proteases, the present invention provides methods of increasing the osteoinductivity of a bone matrix, or the chondrogenic activity of a cartilage repair matrix, by including a lipase, a glycosidase, or any enzyme that generates peptides or protein fragments having the desired activity, in the matrix. In related embodiments, instead of a contacting the bone or cartilage matrix with a protease, the bone or cartilage matrix is contacted with a chemical or condition that generates active peptide or protein fragments. For example, chemicals such as catalytic chemicals or reactive chemicals, such as acids, bases, cyanogen

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bromide, etc., are known to digest or degrade proteins. Conditions that may cause protein break down, resulting in the generation of active peptides and protein fragment domains include, for example, changes in temperature (e.g., heat or cold) and pH (e.g., acidic or basic conditions). Protein digestion or degradation that occurs *via* a protease is referred to herein as specific degradation, whereas protein digestion degradation that occurs *via* a chemical or condition is referred to herein as non-specific degradation. Those skilled in the art will appreciate that any protease, chemical, or condition that generates peptides or protein fragments having osteoinductivity or chondrogenic activity in bone or cartilage matrices, respectively, can be used in the present invention.

[60] In certain preferred embodiments, peptides or protein fragments are derived from longer proteins by contacting the proteins with one or more proteases to generate peptides or protein fragments having osteogenic and/or chrondrogenic properties.

[61] In other preferred embodiments, the present invention provides methods of increasing the osteoinductivity of bone matrix by contacting a bone matrix with at least one protease that selectively degrades inhibitors of osteogenic activity. According to these embodiments, the resulting bone matrix has an increased osteoinductivity, compared to a bone matrix lacking the protease, because inhibition of osteoinductivity is blocked. This increases the overall osteogenic potential of the bone matrix.

[62] In related embodiments, the present invention provides methods of increasing the chondrogenic activity of a cartilage repair matrix by contacting the cartilage repair matrix with at least one protease that selectively degrades inhibitors of chondrogenic activity, wherein the result is a cartilage repair matrix having improved cartilage formation compared to a cartilage repair matrix not contacted with the protease. By blocking the inhibition of chondrogenic factors, the overall chondrogenic activity of the matrix is increased.

[63] As will be appreciated by those skilled in the art, factors having osteoinductive and/or chondrogenic activity can be inhibited by a variety of mechanisms including proteolytic degradation, binding or sequestration of the factor, etc. In various embodiments of the invention, any chemical or condition that blocks inhibition of osteoinductive or chondrogenic factors may be used in the present invention to increase the overall osteogenic or chondrogenic potential of a bone or cartilage matrix. In certain embodiments of the invention, a first protease that cleaves a protein to generate active peptides or protein fragments can be used in combination with a

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second protease (or a chemical or condition) that blocks inhibition of one or more osteoinductive or chondrogenic factors. For example, the second protease may cleave or degrade a protein that would otherwise sequester an osteoinductive or chondrogenic factor, thereby releasing the factor and allowing it to become active. As another example, a protease inhibitor that inhibits a protease known to degrade an osteoinductive or chondrogenic factor can be included in the matrix. In other preferred embodiments, certain chemicals or conditions may be used in combination to both generate osteoinductive or chondrogenic peptides or protein fragments and block inhibition of such factors in bone and cartilage matrices. By combining the methods, as described herein, the osteoinductivity or chondrogenic activity of a bone or cartilage matrix, respectively may be further increased.

[64] In other preferred embodiments, the present invention provides osteoinductive bone matrix compositions for implantation into a bone defect site. In certain preferred embodiments of the invention the compositions comprise a bone matrix including partially demineralized, demineralized, deorganified, or anorganic bone matrix, or a combination there of, treated with at least one protease. The protease causes cleavage of inactive proteins and/or proteins that are susceptible to cleavage or degradation in the body, to generate osteoinductive peptides or protein fragments. The osteoinductive peptide or protein fragments have increased osteoinductivity relative to the uncleaved protein(s) and/or are less susceptible to cleavage or degradation. The resulting bone matrix has an increased osteoinductivity compared to an untreated matrix, resulting in improved bone formation. In other embodiments of the invention, a chemical or condition that causes degradation or digestion of inactive proteins and/or cleavage of proteins that are susceptible to cleavage or degradation in the body, is used in order to generate osteoinductive peptides or protein fragments, resulting in a bone matrix having increased osteoinductivity. In yet other preferred embodiments, the bone repair matrix may include proteases or chemicals that generate osteoinductive peptides or protein fragments in combination with proteases or chemicals that block inhibitor(s) of osteoinductive factors. Such combined formulations result in a further increase in osteoinductivity of the bone matrix.

[65] Cartilage repair matrix compositions are also provided for implantation into a cartilage defect site, which include a cartilage repair matrix treated with at least one protease that causes cleavage of inactive proteins and/or cleavage of proteins that are susceptible to cleavage or degradation in the body, to generate chondrogenic peptides or protein fragments that have

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increased activity relative to the uncleaved protein(s) and/or are less susceptible to cleavage or degradation. The treated cartilage repair matrix has increased chondrogenic activity compared to an untreated cartilage repair matrix, resulting in improved cartilage formation. The cartilage repair matrix compositions may further include one or more chemicals or conditions that increase or replace the function of the protease in generating peptides and protein fragments having chondrogenic activity. In other preferred embodiments, as recited herein, proteases, chemicals, or conditions that block inhibitors of chondrogenic activity may also be included. Such combined formulations result in a further increase in chondogenic activity of the cartilage repair matrix.

[66] For example, the peptide DHLSDNYTLDHDRAIH (Link N), cleaved from the N-terminus of the link protein component of cartilage proteoglycan aggregates by the action of stromelysin, can act as a growth factor and stimulate synthesis of proteoglycans and collagen in articular cartilage (McKenna, Liu, Sansom and Dean (1998) Arthritis Rheum. 41, 157-161). Thus certain cartilage repair matrices of the invention include stromelysin, which acts to increase the amount of this peptide in the matrix. It has also been shown that two major proteases, an initial serine proteinase followed by a metalloproteinase, act in sequence to degrade this peptide (Dean MF and Sansom P., *Biochem J.* 2000 Jul 15;349(Pt 2):473-9). Therefore in certain embodiments of the invention inhibitor(s) of one or both of these proteases are included in the matrix in order to reduce degradation of the cartilage growth factor Link N peptide.

[67] In other preferred embodiments, the present invention provides bone matrices containing one or more peptides or protein fragments having osteoinductive activity. The bone matrix including the osteoinductive peptides or protein fragments has enhanced osteoinductive properties and improved bone formation ability compared to a bone matrix lacking the peptides or protein fragments. In related embodiments, the present invention provides cartilage repair matrices containing peptides or protein fragments that are capable of enhancing the chondrogenic activity of the cartilage repair matrix, resulting in improved cartilage formation ability compared to a composition without the peptide or protein fragment.

[68] A variety of peptides and protein fragments can be generated or included in the bone and cartilage matrices of the present invention, as long as they enhance the osteoinductive or chondrogenic activity of the matrix. In certain preferred embodiments, the peptides and protein fragments can be endogenous and/or exogenous to the matrix. Furthermore, the peptides and

protein fragments used in the invention are derived from growth factors such as, for example, bone morphogenic proteins (e.g., BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8, BMP9, BMP10, BMP11, BMP12, and BMP13), transforming growth factors (TGF) (e.g., from the TGF-β superfamily, e.g., TGF–β), osteogenic factors, vascularizing factors, macrophage colony stimulating factor (MCSF), insulin-like growth factor (e.g., IGF-1), angiogenic factors (e.g., vascular endothelial growth factor (e.g., VGEF), osteonectin, alpha-2-HS glycoprotein, osteocalcin, osteopontin, *etc.* In other preferred embodiments the peptides or protein fragments can be derived from any other collagenous or non-collagenous (for example, matrix GLA protein *etc.*) proteins alone or in combination. In other preferred embodiments the peptides and protein fragments are derived from cell signaling molecules, transcription factors, or hormones. In yet other preferred embodiments the targets of the proteins, chemicals, or conditions of the invention are growth factors agonists. There are also likely to be other unnamed or undiscovered osteoinductive and chondrogenic factors present in bone and cartilage matrix compositions.

[69] In certain preferred embodiments, the peptides and protein fragments can be added in combination with any of a variety of growth factor agonists (e.g., bone morphogenic agonists such as noggin) and bioactive agents (e.g., anti- or pro-inflammatory modulators or drugs), as described herein below. Certain preferred bioactive agents include hormones such as estrogen and parathyroid hormone or other endogenously produced molecules such as prostaglandins. For example, stimulation of the estrogen receptor–α stimulates the adaptive response of bone to mechanical loading, suggesting that estrogen may increase osteoinductivity of a bone matrix (see Lee et al., *Nature*, (July, 2003) 424:389).

[70] Synthetic compounds that have osteoinductive or chondrogenic activity may also be included in the present bone and cartilage formulations. For example, agonists of EP2 receptor selective prostaglandin E2, such as the nonpeptidyl CP,533,536, have been shown to induce bone healing, making such molecules prime candidates to include in, e.g., demineralized bone matrices *etc*, see Paralkar et al., *Proc. Natl, Acad. Sci., USA*, (May, 2003) 100(11): 6736-6740; Seppa, *Science News*, (May 2003) 163:309-310). Those skilled in the art will appreciate that other synthetic molecules having osteogenic or chondrogenic activity could also be included in a bone or cartilage matrix. Means of identifying such synthetic molecules are described in, for example, Paralkar et al., *supra*, or Seppa, *supra*. Other methods of identifying such synthetic molecule are known in the art.

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[71] Development of a vasculature around the implant site may also be important to forming new bone and/or cartilage tissues. Angiogenesis may be an important contributing factor for the replacement of new bone and cartilage tissues. In certain preferred embodiments of the invention, angiogenesis is promoted so that blood vessels are formed at the site to allow efficient transport of oxygen and other nutrients and growth factors to the developing bone or cartilage tissue. In particularly preferred embodiments, angiogenesis promoting factors are included in the bone or cartilage matrix to increase angiogenesis in that region. For example, class 3 semaphorins, e.g., SEMA3, controls vascular morphogenesis by inhibiting integrin function in the vascular system (Serini et al., *Nature*, (July 2003) 424:391-397, incorporated herein by reference) and may be included in the matrix.

[72] In certain preferred embodiments of the present invention, cytokine inhibitors are added to the cartilage matrix to improve bone and cartilage repair. The presence of cytokines, particularly in cartilage, is associated with abnormal extracellular matrix remodeling and loss. A variety of cytokines may have this effect, including, interleukins such as members of the interleukin-1 (IL-1) family of cytokines (IL-1α, IL-1β, IL-18, and IL-1ra), see Lotz, *Clinical Orthopaedics* and Related Research, (2001) 391S: S108-S115). Transforming growth factor-β can compensate for the catabolic effects of IL-1 and enhance cartilage repair, (see van den Berg et al., *Clinical Orthopaedics and Related Research*, (2001) 391S:S2244-S250r). In addition, it has been shown that transforming growth factor-β and bone morphogenetic protein-2 induce chondrophyte formation at the margins of arthritic joints, contributing to spontaneous cartilage repair and chondrophyte formation in arthritic joints (van den Berg et al., *supra*). Thus, in certain preferred embodiments of the invention, cartilage matrices include cytokine inhibitors such as transforming growth factor-β and bone morphogenetic protein-2.

[73] In other preferred embodiments, the peptides or protein fragments of the invention, whether generated in the matrix or added to the matrix mechanically, are covalently or non-covalently attached to the matrix using standard methods, which are well known in the art. Those skilled in the art will further appreciate that in some cases this may require modification of the peptide or protein fragment with a chemical entity or group.

[74] Other preferred embodiments of the present invention provide methods of preparing a bone matrix composition, which include 1) providing the bone matrix, and 2) introducing into

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and/or adsorbing onto the bone matrix peptides or protein fragments that are capable of enhancing the osteoinductivity of the bone matrix, resulting in improved bone formation ability, as compared to a composition without the peptides or protein fragments. Similarly, these methods applied to cartilage include 1) providing a cartilage repair matrix, and 2) introducing into and/or adsorbing onto the cartilage repair matrix peptides or protein fragments that are capable of enhancing the chondrogenic activity of the cartilage repair matrix, resulting in improved cartilage formation ability compared to a composition without the peptides and protein fragments.

[75] In another embodiment, the present invention provides methods of treating a bone or cartilage defect, by implanting the inventive bone or cartilage matrix compositions into an animal, preferably a human, at the site of the bone or cartilage defect. In one particularly preferred embodiment, a demineralized cortical cancellous bone in the shape of fibers is treated with the proprotein convertase, furin, which specifically activates BMPs, as shown schematically in Figure 1 (right side). The matrix is contacted with furin, BMPs are activated and furin and other unwanted components are then optionally washed away from the matrix. Any one of the type of the matrix, the shape of the matrix, the type of treatment, and the specific peptides and proteins activated, as well as an optional inactivation step, may be substituted, with another, as described herein.

[76] A variety of post treatment steps can be used to eliminate the protease and/or unwanted components from the bone or cartilage matrix besides a washing step. In certain embodiments of the invention the protease and/or unwanted component(s) are inactivated by heat, chemicals, or quenching with excess substrate. In other embodiments, the protease and/or unwanted component(s) are not inactivated or removed from the bone or cartilage matrix.

III. Matrix Treatment to Increase Osteoinductive, Osteoconductive, or Chondrogenic Activity

[77] In certain preferred embodiments of the invention, one or more enzymes, such as proteases, lipases, glycosidases, are added to the matrix to activate the osteoinductive or chondrogenic factors already present (e.g., to convert one or more factors from an inactive to an active form or from an active form to a more active form, or from a form that is susceptible to degradation to a form that is less susceptible to degradation, e.g., a form that has a longer half-life). In other preferred embodiments, one or more chemical treatments or application of a

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condition with or without simultaneous enzymatic treatment activates osteoinductive or chondrogenic factors. Many of the growth factors responsible for the osteoinductive or chondrogenic activity of the matrix exist in cryptic form, in the matrix, until activated. Activation can involve the change of a pre or pro function of a factor, or release of the function from a second factor or entity, which binds to the first growth factor. For example, proteolytic cleavage results in separation of the inactive proprotein (e.g., a proprotein from the TGF superfamily of proproteins, e.g., TGF-β) and release of an active, mature peptide. As proteins of bone and cartilage matrices degrade naturally or artificially, they break down into peptides and protein fragments that contain active domains and function as receptor ligands and signal transducers in bone and cartilage growth signaling pathways. The present invention promotes these reactions for the enhancement of osteoinductive and chondrogenic signaling in the bone and cartilage matrices of the invention.

[78] The treatments of the invention may be similar to processes that naturally occur in the body. As is well known in the art, many proteins undergo proteolytic cleavage following translation. The simplest form of this is the removal of the initiation methionine. Many proteins are synthesized as inactive precursors that are activated under proper physiological conditions by limited proteolysis. Pancreatic enzymes and enzymes involved in clotting are examples of the latter. Inactive precursor proteins that are activated by removal of polypeptides are termed, proproteins.

[79] Proteins that are membrane bound or are destined for excretion are synthesized by ribosomes associated with the membranes of the endoplasmic reticulum (ER). The ER associated with ribosomes is termed rough ER (RER). This class of proteins all contain an N-terminus termed a signal sequence or signal peptide. The signal peptide is usually 13-36 predominantly hydrophobic residues. The signal peptide is recognized by a multi-protein complex termed the signal recognition particle (SRP). This signal peptide is removed following passage through the endoplasmic reticulum membrane. The removal of the signal peptide is catalyzed by signal peptidase. Proteins that contain a signal peptide are called preproteins to distinguish them from proproteins. However, some proteins that are destined for secretion are also further proteolyzed following secretion and, therefore contain pro sequences. This class of proteins is termed preproproteins.

[80] A complex example of post-translational processing of a preproprotein is the cleavage of prepro-opiomelanocortin (POMC) synthesized in the pituitary. This preproprotein undergoes complex cleavages, the pathway of which differs depending upon the cellular location of POMC synthesis. Another is example of a preproprotein is insulin. Since insulin is secreted from the pancreas it has a prepeptide. Following cleavage of the 24 amino acid signal peptide the protein folds into proinsulin. Proinsulin is further cleaved yielding active insulin which is composed of two peptide chains linked together through disulfide bonds. Still other proteins (of the enzyme class) are synthesized as inactive precursors called zymogens. Zymogens are activated by proteolytic cleavage such as is the situation for several proteins of the blood clotting cascade. Ini certain embodiments, the present invention may mimic and/or enhance certain naturally occurring processes that result in production of active molecules from inactive precursors.

[81] In preferred embodiments, the invention provides highly osteoinductive bone matrices by treating bone and cartilage preparations of various forms with enzymes, chemicals, or conditions, which process any immature osteoinductive proproteins into their active mature forms. Similarly, the invention further provides highly chondrogenic cartilage repair matrices by treating cartilage grafts with proteases, chemicals, or conditions that process any immature chondrogenic factors into their active mature forms.

[82] A wide variety of agents, selected from proteases, chemicals, and conditions can be used in the present invention to generate osteoinductive peptides and protein fragments, and these are well known in the art. The proteases, chemicals, and conditions of the present can be site specific, amino acid site specific, protein specific, semi-site-specific, lipid specific, or sugar specific, *etc.*

[83] The proteases of the invention may be obtained from endogenous, exogenous, autogenic (autologous), allogenic, or xenogenic sources. According to the present invention, the peptides or protein fragments generated by these proteases and other treatments may be purified by conventional methods (see, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, N.Y.; Ausubel et al. "*Current Protocols in Molecular Biology*, Greene Publishing Associates, New York, V 1 & 2 1996), or used in the bone or cartilage repair matrix as unpurified preparations as long as the peptides or protein fragments maintain their osteoinductive or chondrogenic activity. Alternatively, the peptides or protein fragments can be synthesized artificially using conventional techniques.

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[84] Certain preferred proteases include members of the proprotein convertase (PPC) family of proteases, such as furin and related proteases. Members of this family of cellular enzymes cleave most prohormones and neuropeptide precursors. Numerous other cellular proteins, some viral proteins, and bacterial toxins that are transported by the constitutive secretory pathway are also targeted for maturation by PCs. Furin and other PC family members share structural similarities which include a heterogeneous ~10 kDa amino-terminal proregion, a highly conserved ~55 kDa subtilisin-like catalytic domain, and carboxyl-terminal domain that is heterogeneous in length and sequence. These enzymes become catalytically active following proregion cleavage within the appropriate cellular compartment.

[85] Furin is the major processing enzyme of the secretory pathway and is localized in the trans-golgi network (van den Ouweland, A. M. W. et al. (1990) Nucl. Acid Res. 18, 664; Steiner, D. F. (1998) Curr. Opin. Chem. Biol. 2, 31-39). Substrates of furin include blood clotting factors, serum proteins and growth factor receptors such as the insulin-like growth factor receptor (Bravo D. A. et al. (1994) J. Biol. Chem. 269, 25830-258373). The minimal cleavage site for furin is Arg-X-X-Arg. However, the enzyme prefers the site Arg-X-(Lys/Arg)-Arg. An additional arginine at the P6 position appears to enhance cleavage (Krysan D. J. et al. (1999) J. Biol. Chem. 274, 23229-23234). Furin is inhibited by EGTA, α1- antitrypsin Portland (Jean, F. et al. (1998) Proc. Natl. Acad. Sci. USA 95, 7293-7298) and polyarginine compounds (Cameron, A. et al. (2000) J. Biol. Chem. 275, 36741-36749).

[86] Furin has been shown to proteolytically process both proTGF and proBMP proteins, for example, proTGF-β and proBMP-4, respectively, resulting in the release of the active mature form for each molecule (Dubois et al., *American Journal of Pathology* (2001) 158(1):305-316; Cui et al., *The Embo Journal* (1998) 17(16):4735-4743; Cui et al., *Genes & Development* (2001) 15:2797-2802, each incorporated by reference herein). Furin has also been shown to cleave BMP-2, BMP-6, and BMP-7. For example, furin cleaves between amino acids 282 and 283 in mature human BMP-2. Newly synthesized human BMP-2 contains a signal sequence (amino acids 1-23), a propeptide (amino acids 24-282), and an active portion (amino acids 283-396). Furin cleaves mature BMP-2 (amino acids 24-396) between amino acids 282 and 283 to release the propeptide and the active molecule.

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[87] In accordance with certain embodiments of the invention treating DBM with PPCs such as furin and/or other proteases, which process immature TGF-β and/or BMP superfamily propeptides into their active mature forms and/or process active or inactive TGF-β and/or BMP superfamily polypeptides into smaller active fragments that are resistant to degradation or inactivation relative to the longer polypeptide, generates a bone matrix with increased osteoinductivity compared to a bone matrix lacking the protease, resulting in improved bone formation. The higher titers of the mature and/or degradation resistant species in these preparations increase the osteoinductive capacity of the bone matrix. Preferably, the activation of active factors and/or the generation of degradation-resistant active fragments within the bone matrix increases the overall osteoinductive activity of the bone matrix, compared to bone matrix lacking a protease.

[88] Proteases such as PPCs can also be applied to cartilage repair matrices to activate peptides and protein fragments having chondrogenic activity. This yields a cartilage repair matrix having increased chondrogenic activity compared to a cartilage repair matrix lacking the protease. The activation of chondrogenic peptides and protein fragments within the cartilage repair matrix increases the overall chondrogenic activity of the matrix and results in improved cartilage formation, compared to a cartilage repair matrix lacking a protease.

[89] According to the present invention, activation of a peptide or protein fragment can be either specific or non-specific. Cleavage of a protein, e.g., with a particular protease to generate active peptides and protein fragments is referred to as specific activation, or specific digestion or degradation. Non-specific activation can occur when protein digestion or degradation is caused by conditions such as changes in temperature or pH.

[90] As disclosed herein, other changes or alterations in a peptides or protein fragment can also result in activation including, for example, conformational change, post-translational modification, a change in tertiary or quaternary structure, release from the matrix, release from a binding protein, *etc.* Such changes can also occur specifically by contact with a specific enzyme or chemical, or non-specifically from changes in temperature or pH. Other non-specific conditions that may be useful in these capacities would be obvious to one skilled in the art.

[91] The present invention provides bone and cartilage grafts along with kits and methods for preparing bone and cartilage grafts having an increased osteoinductive or chondrogenic activity, respectively, compared to grafts containing longer proteins In general, the invention provides

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methods of treating bone and cartilage matrices to activate inactive factors that are already present in the bone or cartilage matrix.

IV. Transcriptional and Post-transcriptional Regulation of Bone or Cartilage Enhancing or Inhibiting Factors

[92] As discussed above in certain embodiments of the invention, cells migrate into the inventive bone or cartilage repair matrices after their implantation into the body. In certain embodiments of the invention cells (either autologous, allogeneic, or xenogeneic) are already present in the matrix prior to implantation, and additional cells may enter the matrix after implantation. In either case, certain of the cells preferably contribute to the development and/or strengthening of the matrix, e.g., via the deposition of new bone and/or cartilage components and/or the reorganization or remodeling of components already present in the matrix or newly synthesized. Other cells may contribute to development of blood vessels, etc.

[93] As mentioned above, agents such as protein fragments, peptides, growth factors, hormones, etc., can influence the biological activity and/or functioning of these cells. It will also be appreciated that certain of the cells may themselves produce molecules such as proteins, hormones, growth factors, chemoattractants, cytokines, etc., that may influence either their own functional activity or that of other cells either in the matrix or elsewhere in the body. Among these molecules are various molecules that act positively to promote proper formation of bone or cartilage. These molecules include bone or cartilage growth factors or factors that inhibit the activity of inhibitors of bone or cartilage formation. Among these positively acting molecules are bone morphogenetic proteins such as BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8, BMP9, BMP10, BMP11, BMP12, and BMP13, transforming growth factors (TGF) such as those from the TGF-β superfamily, e.g., TGF–β, osteogenic factors, vascularizing factors, macrophage colony stimulating factor (MCSF), insulin-like growth factor (e.g., IGF-1), angiogenic factors (e.g., vascular endothelial growth factor (e.g., VGEF), osteonectin, alpha-2-HS glycoprotein, osteocalcin, osteopontin, matrix GLA protein *etc.* For purposes of the present description, nucleic acids or proteins whose expression positively influences formation, development, or repair of bone or cartilage, such as bone or cartilage growth factors, will be referred to as bone/cartilage enhancing factors (BCEF). Cells may also produce negatively acting molecules, e.g., molecules whose presence interferes with or reduces proper formation of bone or cartilage.

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Certain cytokines may have this effect, including interleukins such as the interleukin-1 (IL-1) family of cytokines (e.g., IL-1α, IL-1β, IL-18, and IL-1ra) and various other bone growth inhibitors (e.g., epidermal growth factor, alpha 2HS glycoprotein, heparin, noggin, chordin, and fetuin). For purposes of the present description, nucleic acids or proteins whose expression negatively influences formation or development of bone or cartilage will be referred to as bone/cartilage inhibitory factors (BCIF).

[94] The inventors have recognized that by modulating the expression of certain BCEF and/or BCIF by cells within the matrix (or elsewhere in the body), it is possible to increase the osteoinductive, osteoconductive, and/or chondrogenic activity of a bone or cartilage repair matrix. Generally it will be desirable to increase expression of BCEF and/or decrease expression of BCIF although it may at times be desirable to decrease expression of BCEF and/or increase expression of BCIF. Accordingly, in certain embodiments of the invention the bone and cartilage repair matrices incorporate any of a variety of agents that influence the biological activity and/or functioning of cells by transcriptional or post-transcriptional regulation of the expression of BCEF and/or BCIF molecules such as those mentioned above.

A. Transcriptional Regulation

[95] Transcriptional or post-transcriptional regulation can be achieved through any of a variety of means. For example, the expression of BCEF and/or BCIF may be altered by incorporating transcription activators or inhibitors within the matrix. Preferably such activators or inhibitors of transcription act specifically to increase or decrease transcription from a promoter that is operably associated with a nucleic acid that encodes a BCEF or BCIF. Small molecules may generally be preferred since they are readily able to enter cells. For example, small molecules that modulate transcription factor activity, e.g., molecules that activate or inhibit transcription factors that enhance or reduce transcription from a promoter that is operably associated with a nucleic acid that encodes a BCEF or BCIF can be used. Examples of such small molecules include naturally occurring hormones such as estrogen and synthetic molecules that act as agonists or antagonists at hormone receptors such as the estrogen receptor. For example estrogens are known to exert their physiological effects on target tissues by interacting with estrogen receptors, ERalpha and ERbeta. The estrogenic compound 17 beta-estradiol (E2) has been shown to increase mouse bone morphogenetic protein (BMP)-2 mRNA by increasing the transcription of the BMP-2 gene, using a mechanism that depends on ERalpha and/or ERbeta

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(Zhou, S., *et al, Mol Endocrinol.* 2003 Jan;17(1):56-66). Methods for identifying small molecule modulators of transcription factor activity are known in the art. Such molecules may act by a number of different mechanisms. For example, they may interact directly with a transcription factor (e.g., by phosphorylating it) or may act on another cellular protein that in turn interacts with a transcription factor. (See, e.g., Mohan R and Heyman RA. *Curr Top Med Chem.* 2003;3(14):1637-47 and Barrie, S.E., *et al., Anal Biochem.* 2003 Sep 1;320(1):66-74 for representative examples.)

[96] Alternately, in certain embodiments of the invention transcription factors themselves (which term is taken to include proteins that negatively regulate transcription by interacting with transcriptional control elements such as promoters or enhancers or by interacting with components of the transcription machinery such as positively acting transcription factors), are delivered to cells. The transcription factors may be encapsulated, e.g., within polymeric delivery vehicles that protect them from degradation and/or provide for controlled release.

[97] In certain embodiments of the invention the transcription factor(s) are provided as fusion proteins that incorporate a transport polypeptide, such as those described in U.S. Patent Nos. 6,316,003; 5,804,604; 5,747,641; and 5,674,980 that enhances transport of the protein into cells. Alternately, nucleic acid(s) that encode transcription factors may be provided. The nucleic acid(s) are taken up by cells and intracellular synthesis of transcription factors then takes place. The nucleic acids may be DNA or RNA and may be provided in single-stranded or double-stranded form. They may be encapsulated, e.g., within polymeric delivery vehicles that protect them from degradation and/or provide for controlled release. The nucleic acids may be provided as conjugates that incorporate a transport polypeptide, as described, for example, in U.S. Patent No. 5,670,617. It is noted that a variety of other peptide molecular transporters are known in the art and may be similarly used. In certain embodiments of the invention the transcription factors or nucleic acids may be modified to incorporate a targeting moiety.

[98] In addition to the use of naturally occurring transcription factors, the invention encompasses the use of engineered DNA binding proteins that recognize and bind to specific nucleotide sequences and modulate (e.g., increase or decrease) the transcription of genes that are operably linked to these nucleotide regions. Design of such transcription modulating proteins is described, for example, in U.S. Patent No. 6,326,166 and 6,410,248. In general, certain of these engineered transcription modulating proteins that increase transcription include a DNA binding

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portion (e.g., a zinc finger domain, homeodomain, or a modified form of the foregoing, etc.) and a transcriptional activation domain capable of activating transcription of a gene linked to a DNA sequence to which the transcription factor can bind (e.g., a Herpes Simplex Virus VP16 activation domain, an NF-.kappa.B p65 activation domain, others transcription activation domains that are also derived from naturally occurring transcription factors, or chimeric or entirely artificial transcription activation domains). Certain engineered transcription modulating proteins decrease or repress transcription of a target gene linked to a nucleotide sequence to which the proteins bind. Such proteins may function in a manner analogous to a classical repressor by binding to a nucleotide sequence and blocking, in whole or part, the otherwise normal functioning of that nucleotide sequence in gene expression, e.g., by preventing binding of an endogenous transcription factor. Certain transcription modulating proteins capable of repressing or inhibiting transcription of a gene linked to a nucleotide sequence to which the chimeric protein binds include chimeric proteins containing a DNA-binding region, characteristic of all chimeric proteins of this invention, and an additional domain, such as a KRAB domain or a ssn-6/TUP-1 or Kruppel-family suppressor domain, capable of inhibiting or repressing the expression of the gene in a cell. In either case, binding of the chimeric protein to the nucleotide sequence linked to the target gene is associated with decreased transcription of the gene.

[99] In certain embodiments of the invention transcription factors (either naturally occurring transcription factors or engineered transcription modulating proteins) are delivered to cells via vectors that contain nucleic acids that encode such factors, operably linked to transcriptional control elements such as promoters, enhancers, etc., suitable for expression of the factors within mammalian cells. In general, a wide variety of promoters and/or enhancers are known in the art and may be used, including viral promoters (e.g., CMV, SV40), constitutive promoters (e.g., actin, tubulin, etc.), inducible promoters (e.g., hormone-inducible, metal-inducible, tetracycline-regulated, etc.), tissue or cell type specific, etc. For example, it may be desirable to utilize promoters and/or enhancers that are known to be active in osteoblasts, osteocytes, chondroblasts, chondrocytes, etc.) Of course a promoter and/or enhancer that is operably associated with the transcription factor in nature can be used. In those embodiments of the invention in which an inducible promoter is used, the inducing agent (e.g., tetracycline) may be incorporated within the matrix or may be delivered to the subject following introduction of the matrix into the body. In

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either case the inducing agent may be encapsulated within a delivery agent that provides controlled release and/or targets the inducing agent to a particular cell type, etc.

[100] In general, the vectors can be DNA vectors, including plasmids containing viral sequences such as retroviral or lentiviral vectors. Alternately, the vectors can be virus vectors, e.g., retrovirus, lentivirus, adenovirus, or adeno-associated virus vectors. In general, a wide variety of vectors that find use in gene therapy applications may be employed to deliver transcription factors to cells within the matrices. Additional materials that enhance uptake of the vectors by cells may also be included, e.g., cationic or non-cationic lipids, cationic polymers, etc. In certain embodiments of the invention the vectors are encapsulated, e.g., in liposomes, within polymeric delivery vehicles, etc. Such delivery agents or the vectors themselves may be modified, e.g., to incorporate targeting moieties. Vectors may also be modified to incorporate agents such as polyethylene glycol (PEG) that act to stabilize the vector. See S.-O. Han, R. I. Mahato, Y. K. Sung, S. W. Kim, "Development of biomaterials for gene therapy", *Molecular Therapy* 2:302317, 2000 for additional discussion of agents that may be used to enhance delivery of vectors to cells within the matrices. In general, the transcription factors, nucleic acids that encode transcription factors, and/or vectors may be incorporated into the matrices in a similar manner to that in which proteases and other active agents are incorporated, as described below.

[101] Accordingly, the invention provides a bone or cartilage repair matrix comprising a transcription modulator such as a transcription activator or inhibitor, wherein the transcription modulator modulates transcription of a BCEF or BCIF. In various embodiments of the invention the transcription modulator is a small molecule, a transcription factor, or an engineered transcription modulating protein. In other embodiments of the invention the transcription modulator is a vector that provides a template for intracellular synthesis of a transcription factor or engineered transcription modulating protein. The invention further encompasses a method of increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising introducing a transcription modulator such as a transcription activator or inhibitor, wherein the transcription modulator modulates transcription of a BCEF or BCIF into the matrix. The transcription modulator can be, for example, a small molecule, a transcription factor, an engineered transcription modulating protein, or a vector that provides a template for intracellular synthesis of a transcription factor or engineered transcription modulating protein.

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[102] In those embodiments of the invention in which cells are incorporated into the matrix (either having been incorporated directly into the matrix prior to introduction of the matrix into the body or delivered to the matrix afterwards), transcriptional regulation of the expression of BCEF or BCIF by the cells may be achieved by delivery of any of the agents described above to the cells *in vitro*, e.g., while the cells are in tissue culture or after they are mixed with matrix components or introduced into the matrix, but prior to their introduction into a subject.

[103] In those embodiments of the invention in which genetically engineered cells are present within the matrix (either having been incorporated directly into the matrix prior to introduction of the matrix into the body or delivered to the matrix afterwards, or via migration of cells into the matrix following implantation) transcriptional regulation of the expression of BCEF or BCIF by the cells may be achieved by modifying the sequence(s) of regulatory elements such as promoters and/or enhancers that are operably linked to the nucleic acid that encodes the BCEF or BCIF. For example, the sequence may be modified to increase or decrease transcription factor binding affinity. Modification may be achieved using methods that are well known in the art, e.g., site-directed mutagenesis, PCR-mediated mutagenesis, etc. Modified sequences may be generated by trial and error, by design, or by directed evolution strategies, etc. The effect of any given modification on transcription factor binding affinity and/or transcription can be tested using standard methods and desirable modifications thereby identified.

[104] The modified sequences may replace the corresponding endogenous sequence (e.g., via homologous recombination). Alternately, the modified sequences may be operably linked to genes that encode the transcription factor and included within a vector such as those described above, which is delivered to cells. Nucleic acid molecules or vectors can be introduced into cells *in vitro*, e.g., via conventional transfection techniques. As used herein, the term "transfection" is intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA or RNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, injection, or electroporation.

[105] Accordingly, the invention provides a method of increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising introducing a cell comprising a modified regulatory sequence into the matrix, wherein the modified regulatory sequence is operably linked to a nucleic acid that provides a template for transcription of a BCEF or BCIF and increases or decreases transcription factor

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binding affinity or transcription relative to a corresponding naturally occurring regulatory sequence lacking such modification. The invention further provides cells, e.g., osteoblasts, osteocytes, osteoclasts, chondroblasts, chondrocytes, etc., comprising such modified regulatory sequences. In addition, the invention features bone repair matrices and cartilage repair matrices into which such cells have been introduced (either by the hand of man or by migration within the body of a subject).

B. Post-transcriptional Regulation

[106] In addition to trancriptional regulation of BCEF and/or BCIF expression, the invention encompasses use of a variety of methods for achieving post-transcriptional control. In particular, nucleic acid molecules such as antisense nucleic acids, ribozymes, or molecules that mediate RNA interference such as short interfering RNA (siRNA), short hairpin RNA (shRNA), or precursors of microRNA (miRNA) can be delivered to cells or expressed intracellularly to achieve post-transcriptional regulation. The latter can be achieved by delivery of vectors containing nucleic acids that provide templates for transcription of the antisense, ribozyme, or RNAi-inducing agents such as siRNA, shRNA, or precursors of miRNA to cells. Antisense, ribozyme, and RNAi-based methods of regulating gene expression will be briefly described below. In general, their use is well known in the art. Typically these agents act to decrease expression of a target transcript, thus they may be more commonly employed to reduce expression of BCIF than to reduce expression of BCEF. However, in certain situations it may be desirable to reduce expression of BCEF, e.g., to prevent a bone or cartilage formation response that would otherwise be too exuberant and/or to "fine-tune" the formation and/or properties of the developing tissue.

[107] *Antisense Agents.* Antisense nucleic acids are generally single-stranded nucleic acids (DNA, RNA, modified DNA, modified RNA, or peptide nucleic acids) complementary to a portion of a target nucleic acid (e.g., an mRNA transcript) and therefore able to bind to the target to form a duplex. Typically they are oligonucleotides that range from 15 to 35 nucleotides in length but may range from 10 up to approximately 50 nucleotides in length. For example, antisense oligonucleotides may block transcription when bound to genomic DNA, inhibit translation when bound to mRNA, and/or lead to degradation of the nucleic acid (e.g., via RNAse H or other RNA-cleaving molecules). Reduction in expression of a BCEF or BDIF may be achieved by the administration of an antisense nucleic acid or peptide nucleic acid comprising

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sequences complementary to those of the mRNA that encodes the polypeptide. Antisense technology and its applications are well known in the art and are described in Phillips, M.I. (ed.) *Antisense Technology*, Methods Enzymol., Volumes 313 and 314, Academic Press, San Diego, 2000, and references mentioned therein. See also Crooke, S. (ed.) "Antisense Drug Technology: Principles, Strategies, and Applications" (1st ed), Marcel Dekker; ISBN: 0824705661; 1st edition (2001) and references therein. Peptide nucleic acids (PNA) are analogs of DNA in which the backbone is a pseudopeptide rather than a sugar. PNAs mimic the behavior of DNA and bind to complementary nucleic acid strands. The neutral backbone of a PNA can result in stronger binding and greater specificity than normally achieved using DNA or RNA. Binding typically reduces or inhibits the function of the target nucleic acid. Peptide nucleic acids and their use are described in Nielsen, P.E. and Egholm, M., (eds.) *Peptide Nucleic Acids: Protocols and Applications* (First Edition), Horizon Scientific Press, 1999.

[108] The invention provides a bone or cartilage repair matrix comprising an antisense nucleic acid that decreases expression of BCEF or BCIF mRNA. The invention further provides a bone or cartilage repair matrix comprising a vector that provides a nucleic acid template for transcription of an antisense nucleic acid that decreases expression of BCEF or BCIF mRNA. The invention further encompasses a method of increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising introducing an antisense nucleic acid that decreases expression of BCEF or BCIF mRNA or a vector that provides a nucleic acid template for transcription of an antisense nucleic acid that decreases expression of BCEF or BCIF mRNA into the matrix.

[109] *Ribozymes.* Ribozymes (catalytic RNA molecules that are capable of cleaving other RNA molecules) represent another approach to reducing gene expression. Such ribozymes can be designed to cleave specific mRNAs corresponding to a gene of interest. Their use is described in U.S. Patent No. 5,972,621, and references therein. Extensive discussion of ribozyme technology and its uses is found in Rossi, J.J., and Duarte, L.C., *Intracellular Ribozyme Applications: Principles and Protocols, Horizon Scientific Press*, 1999.

[110] The invention provides a bone or cartilage repair matrix comprising a ribozyme designed to cleave BCEF or BCIF mRNA. The invention further provides a bone or cartilage repair matrix comprising a vector that provides a nucleic acid template for transcription of a ribozyme designed to cleave BCEF or BCIF mRNA. The invention further encompasses a method of

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increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising introducing a ribozyme designed to cleave BCEF or BCIF mRNA or a vector that provides a nucleic acid template for transcription of a ribozyme designed to cleave BCEF or BCIF mRNA into the matrix.

[111] *RNA Interference.* RNA interference (RNAi) is a mechanism of post-transcriptional gene silencing mediated by double-stranded RNA (dsRNA), which is distinct from the antisense and ribozyme-based approaches described above. dsRNA molecules are believed to direct sequence-specific degradation of mRNA that contain regions complementary to one strand (the antisense strand) of the dsRNA in cells of various types after first undergoing processing by an RNase III-like enzyme called Dicer (Bernstein et al., *Nature* 409:363, 2001) into smaller dsRNA molecules. These molecules comprise two 21 nt strands, each of which has a 5' phosphate group and a 3' hydroxyl, and includes a 19 nt region precisely complementary with the other strand, so that there is a 19 nt duplex region flanked by 2 nt-3' overhangs. RNAi is thus mediated by short interfering RNAs (siRNA), which typically comprise a double-stranded region approximately 19 nucleotides in length with 1-2 nucleotide 3' overhangs on each strand, resulting in a total length of between approximately 21 and 23 nucleotides. In mammalian cells, dsRNA longer than approximately 30 nucleotides typically induces nonspecific mRNA degradation via the interferon response. However, the presence of siRNA in mammalian cells, rather than inducing the interferon response, results in sequence-specific gene silencing.

[112] RNAi can also be mediated by molecules referred to as short hairpin RNAs (shRNA), which are single RNA molecules comprising at least two complementary portions capable of self-hybridizing to form a duplex structure sufficiently long to mediate RNAi (typically at least 19 base pairs in length), and a loop, typically between approximately 1 and 10 nucleotides in length and more commonly between 4 and 8 nucleotides in length that connects the two nucleotides that form the last nucleotide pair at one end of the duplex structure. shRNAs are thought to be processed into siRNAs by the conserved cellular RNAi machinery. Thus shRNAs are precursors of siRNAs and are similarly capable of inhibiting expression of a target transcript.

[113] In addition, RNAi can be mediated by single-stranded RNA molecules referred to as microRNAs, in a process that is thought to involve translational repression but is distinct from antisense-mediated translational repression. These microRNAs can be produced by cleavage of longer hairpin structures similar to shRNAs, except that the miRNA precursors typically include

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one or more mismatches, bulges, or inner loops within the stem and also exhibit less than perfect complementarity with the target transcript. For example, in addition to the siRNA and shRNA structures described above, Dicer processing of ~70 nucleotide hairpin precursors with imperfect duplex structures, i.e., duplexes that are interrupted by one or more mismatches, bulges, or inner loops within the stem of the hairpin leads to the production of RNAs that are believed to hybridize within the 3' UTR of a target mRNA and repress translation. See, e.g., Lagos-Quintana, M. et al., *Science*, 294, 853-858, 2001; Pasquinelli, A., *Trends in Genetics*, 18(4), 171-173, 2002, and references in the foregoing two articles for discussion of miRNAs and their mechanisms of silencing.

[114] siRNAs and shRNAs have been shown to downregulate gene expression when transferred into mammalian cells by such methods as transfection, electroporation, or microinjection, or when expressed in cells via any of a variety of plasmid-based approaches. RNA interference mediated by siRNA, shRNA, and or miRNA and considerations for selection of effective RNAi mediating agents is reviewed extensively in McManus, M. and Sharp, P., *Nature Reviews Genetics*, 3: 737-747, and in Dykxhoorn, D.M., *et al., Nature Reviews Molecular Cell Biology*, 4: 457-467, 2003. Tuschl, T., *Nat. Biotechnol.*, 20: 446-448, May 2002. See also Yu, J., et al., *Proc. Natl. Acad. Sci.*, 99(9), 6047-6052 (2002); Sui, G., et al., *Proc. Natl. Acad. Sci.*, 99(8), 5515-5520 (2002); Paddison, P., et al., *Genes and Dev.*, 16, 948-958 (2002); Brummelkamp, T., et al., *Science*, 296, 550-553 (2002); Miyagashi, M. and Taira, K., *Nat. Biotech.*, 20, 497-500 (2002); Paul, C., et al., *Nat. Biotech.*, 20, 505-508 (2002). A number of variations in structure, length, number of mismatches, size of loop, identity of nucleotides in overhangs, etc., are consistent with effective RNAi-mediated gene silencing. For example, one or more mismatches between the target mRNA and the complementary portion of the siRNA or shRNA may still be compatible with effective silencing.

[115] RNAi-mediating agents such as siRNA or shRNA molecules may be generated by intracellular transcription of small RNA molecules, which may be followed by intracellular processing events. For example, intracellular transcription may be achieved by cloning templates into RNA polymerase III transcription units, e.g., under control of a U6 or H1 promoter. Promoters that direct transcription by RNA polymerase I or RNA polymerase II can also be used. In one approach for intracellular synthesis of siRNA, sense and antisense strands are transcribed from individual promoters, which may be on the same construct. The promoters

may be in opposite orientation so that they drive transcription from a single template, or they may direct synthesis from different templates. However, it may be preferable to express a single RNA molecule that self-hybridizes to form a hairpin RNA that is then cleaved by Dicer within the cell.

[116] In certain embodiments of the invention RNAi is used to decrease expression of one or more BCEFs or BCIFs, e.g., by incorporating RNAi-mediating agents or vectors that provide templates for their synthesis into the matrix, or by delivering the agents or vectors to cells that are introduced into the matrix. Accordingly, the invention provides bone repair matrices and cartilage repair matrices comprising an RNAi-mediating agent that decreases expression of BCEF or BCIF mRNA when present within a cell. The invention further provides a bone or cartilage repair matrix comprising a vector that provides a nucleic acid template for transcription of an RNAi-mediating agent that decreases expression of BCEF or BCIF mRNA when present within a cell. The invention further encompasses a method of increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising introducing an RNAi-mediating agent that decreases expression of BCEF or BCIF mRNA or a vector that provides a nucleic acid template for transcription of an RNAi-mediating agent that decreases expression of BCEF or BCIF mRNA into the matrix.

[117] *Synthesis and Modification.* It may advantageous to employ various nucleotide modifications and analogs to confer desirable properties on an antisense nucleic acid, ribozyme, siRNA, or shRNA, etc. Numerous nucleotide analogs and nucleotide modifications are known in the art, and their effect on properties such as hybridization and nuclease resistance has been explored. For example, various modifications to the base, sugar and internucleoside linkage have been introduced into oligonucleotides at selected positions, and the resultant effect relative to the unmodified oligonucleotide compared. A number of modifications have been shown to alter one or more aspects of the oligonucleotide such as its ability to hybridize to a complementary nucleic acid, its stability, etc. For example, useful 2'-modifications include halo, alkoxy and allyloxy groups. US patent numbers 6,403,779; 6,399,754; 6,225,460; 6,127,533; 6,031,086; 6,005,087; 5,977,089, and references therein disclose a wide variety of nucleotide analogs and modifications that may be of use in the practice of the present invention. See also Crooke, S. (ed.), referenced above, and references therein. As will be appreciated by one of ordinary skill in the art, analogs and modifications may be tested using, e.g., the assays described

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herein or other appropriate assays, in order to select those that effectively reduce expression of the target nucleic acid.

[118] In certain embodiments of the invention the analog or modification results in a nucleic acid with increased absorbability (e.g., increased absorbability across a mucus layer, increased oral absorption, etc.), increased stability in the blood stream or within cells, increased ability to cross cell membranes, etc. The invention encompasses antisense nucleic acids, ribozymes, siRNAs, or shRNAs incorporating any of these or other known useful nucleic acid modifications and/or analogs.

[119] Antisense RNAs, ribozymes, siRNAs or shRNAs may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemical synthesis such as solid phase phosphoramidite chemical synthesis. In the case of siRNAs or shRNAs, the structure may be stabilized, for example by including nucleotide analogs at one or more free strand ends in order to reduce digestion, e.g., by exonucleases. This may also be accomplished by the use of deoxy residues at the ends, e.g., by employing dTdT overhangs at one or both 3' ends of an siRNA. Alternatively, antisense, ribozyme, siRNA or shRNA molecules may be generated by *in vitro* transcription of DNA sequences encoding the relevant molecule. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7, T3, or SP6.

[120] Antisense, ribozyme, or RNAi-mediating molecules may be introduced into cells by any of a variety of methods. For instance, antisense, ribozyme, siRNA, or shRNA molecules or vectors encoding them can be introduced into cells via conventional transformation or transfection techniques such as those described above. Vectors (e.g., of any of the types described above) that direct *in vivo* synthesis of antisense, ribozyme, siRNA, or shRNA molecules constitutively or inducibly can be introduced into cells similarly.

[121] In general, the ability of any of the foregoing approaches to increase or decrease expression of a BCEF or BCIF may readily be tested using various methods known in the art. Such assays may be performed in tissue culture systems or in test animals. For example, mRNA expression may be evaluated using Northern blots, RT-PCR, including quantitative RT-PCR, microarray analysis, etc. Protein expression may be evaluated using Western blots, immunoassays, functional assays for protein activity, etc. Reporter-based systems can also be used.

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[122] It is noted that although certain embodiments of the invention encompass cells that may be introduced into or present within a human being, it is to be understood that the claims of the invention do not encompass human beings or portions thereof.

V. Bone and Cartilage Matrices and Matrix Compositions

[123] A variety of tissue types may be subject to regeneration using matrix preparations of the present invention. Several non-limiting examples include cortical bone, cancellous bone, cortical-cancellous bone, cartilage, perichondrium, and perostium, *etc.* Those skilled in the art will appreciate that the shapes that the matrices of the present invention can take can vary depending on the defect they are meant to repair. Some exemplary matrices, described in detail below, include whole matrices, chips, fibers, powders particles, rods, strings, sheets, weaves, solids, cones, discs, wedges etc. Matrices of any tissue type or shape can be exposed to a protease, chemical, or condition of the invention to generate osteoinductive or chondrogenic peptides and protein fragments. Furthermore, it will be appreciated that matrices of any tissue type or shape can be adsorbed with the osteogenic and or chondrogenic peptides and protein fragments of the invention.

[124] Demineralized bone matrix preparations have been used for many years in orthopaedic medicine to promote the formation of bone. For example, demineralized bone matrices have found use in the repair of fractures, congenital bone defects, iatrogenic bone defects, in the fusion of vertebrae, in joint replacement surgery, and in treating bone destruction due to underlying disease such as rheumatoid arthritis. Demineralized bone matrices are thought to promote bone formation *in vivo* by osteoconductive and osteoinductive processes. Osteoconduction occurs if the implanted material serves as a scaffold for the support of new bone growth. Osteoconduction is particularly significant when bone growth is desired across a large or "critical size" defect, across which bone healing would proceed only slowly or not at all. It is generally believed that the osteoconductive properties of demineralized bone matrix preparations are provided by the actual shape and coherence of the implant. Thus demineralized bone matrix compositions including entangled fibers tend to have superior osteoconductive properties as compared to less fibrous, more granular preparations. Stabilizing agents, which tend to preserve the shape and/or coherence of the demineralized bone matrix substituent, can lead to better bone forming properties.

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[125] Any of a variety of demineralized bone matrix preparations may be utilized in the practice of the present invention. Demineralized bone matrix prepared by any method may be employed including particulate or fiber-based preparations, mixtures of fiber and particulate preparations, fully or partially demineralized preparations, mixtures of fully and partially demineralized preparations, including surface demineralized preparations as described by Gertzman et al. (U.S. Patent 6,326,018, issued December 4, 2001; Reddi et al., *Proc. Natl. Acad. Sci. USA* (1972) 69:1601-1605; Lewandrowski et al., *Clin. Ortho. Rel. Res.*, (1995) 317:254-262; Lewandroski et al., *J. Biomed. Mater. Res.* (1996) 31:365-372; Lewandrowski et al. *Calcified Tiss. Int.*, (1997) 61:294-297; Lewandrowski et al., *J. Ortho. Res.* (1997) 15:748-756, incorporated herein by reference). Preferred demineralized bone matrix compositions are described by Dowd et al., U.S. Patent 5,507,813, which is incorporated herein by reference.

[126] In a one preferred demineralization procedure, the implant is subjected to an acid demineralization step followed by a defatting/disinfecting step. The implant is immersed in acid over time to effect demineralization. Acids that can be employed in this step include inorganic acids such as hydrochloric acid and as well as organic acids such as formic acid, acetic acid, peracetic acid, citric acid, propionic acid, etc. The depth of demineralization into the bone surface can be controlled by adjusting the treatment time, temperature of the demineralizing solution, concentration of the demineralizing solution, and agitation intensity during treatment.

[127] The demineralized implant is rinsed with sterile water and/or buffered solution(s) to remove residual amounts of acid and thereby raise the pH. A preferred defatting/disinfectant solution is an aqueous solution of ethanol, the ethanol being a good solvent for lipids and the water being a good hydrophilic carrier to enable the solution to penetrate more deeply into the bone particles. The aqueous ethanol solution also disinfects the bone by killing vegetative microorganisms and viruses. Ordinarily, at least about 10 to 40 percent by weight of water (i.e., about 60 to 90 weight percent of defatting agent such as alcohol) should be present in the defatting disinfecting solution to produce optimal lipid removal and disinfection within the shortest period of time. The preferred concentration range of the defatting solution is from about 60 to about 85 weight percent alcohol and most preferably about 70 weight percent alcohol.

[128] In addition to the demineralizing step, the bone is optionally subjected to a configuring step to form the implant described herein. The configuring step can be employed using conventional equipment known to those skilled in the art to produce a wide variety of

geometries, e.g., concave or convex surfaces, stepped surfaces, cylindrical dowels, wedges, blocks, screws, and the like. A surgically implantable material fabricated from elongated bone particles that have been demineralized, which may be shaped as a sheet, and processes for fabricating shaped materials from demineralized bone particles are disclosed in U.S. Patent Nos. 5,507,813 and 6,436,138, respectively, the contents of which are incorporated by reference herein. Suitable sheets included those sold under the trade name Grafton®Flex, which must be wetted/hydrated prior to use in order to render them useful for implantation. Such sheets have recently been reported as effective in seeding human bone marrow stromal cells (BMSCs), which may be useful in the repair of large bone defects (see, e.g., Kasten, et al., "Comparison of Human Bone Marrow Stromal Cells Seeded on Calcium-Deficient Hydroxyapatite, Beta-tricalcium Phosphate and Demineralized Bone Matrix", *Biomaterials*, 24(15):2593-603, 2003). Also useful are demineralized bone and other matrix preparations comprising additives or carriers such as binders, fillers, plasticizers, wetting agents, surface active agents, biostatic acents, biocidal agents, and the like. Some exemplary additives and carriers include, polyhydroxyl compounds, polysaccharides, glycosaminoglycan proteins, nucleic acids, polymers, polaxomers, resins, clays, calcium salts, and/or derivatives thereof.

[129] The bone used in creating the bone matrix may be obtained from any source of living or dead tissue. Often, it will be preferred that the source of bone be matched to the eventual recipient of the inventive composition. At a minimum, it is often desirable that the donor and recipient are of the same species, though even xenogenic sources are permitted.

[130] Once a bone sample is obtained, it is milled, ground, pulverized, or otherwise reduced to particulate form. The bone sample is then generally shaped to fit an implant site. Following particulation, the demineralized bone matrix is treated to remove mineral from the bone. While hydrochloric acid is the industry-recognized demineralization agent of choice, the literature contains numerous reports of methods for preparing demineralized bone matrices (see, for example, Russell et al., *Orthopaedics* 22(5):524-531, May 1999; incorporated herein by reference). For the purposes of the present invention, any material that provides scaffolding containing active osteoinductive factors is considered demineralized bone matrix. The demineralized bone matrix may be prepared by methods known in the art or by other methods that can be developed by those of ordinary skill in the art without undue experimentation. In some instances, large fragments or even whole bone may be demineralized, and then particulated

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following demineralization. Demineralized bone prepared in this way is within the scope of the invention.

[131] As mentioned above, osteoinductive peptides and protein fragments can be activated within a bone or cartilage matrix specifically (e.g., digestion with a protease) and non-specific conditions (e.g., temperature, pH, *etc.*). In one preferred embodiment, peptides and proteins fragments are activated specifically by digestion with a particular protease. Exemplary proteases that may activate the osteoinductive enzymes of the invention include, acid proteases, serine proteases, metalloproteases, cysteine proteases, glyconases, and glycosidases. Particularly useful proteases are those stable and effective in acidic conditions.

[132] Growth factor binding proteins are specific regulatory factors that can play a major role in regulating the activity of peptides and protein fragments. Virtually every extracellular matrix growth factor is know to be associated with a binding protein that regulates its activity. Typical growth factor binding proteins include but are not limited to noggin, chrondin, follistatin, TGF-β binding protein, and insulin-like growth factor binding proteins. According to the invention, growth factor binding proteins can be used to regulate the activity of peptides and protein fragments having osteoinductive activity.

[133] Cartilage is an avascular tissue composed of 5-10% by weight of living cells. There are three major types of cartilage in the body: hyaline, also known as articular cartilage; fibrocartilage; and elastic cartilage. Articular cartilage covers the epiphyses of the bone and, in synovial joints, lies within a fluid filled capsule. Articular cartilage is load-bearing tissue that distributes forces across joint surfaces, protects the more rigid underlying bone, and provides smooth articulation and bending of the joints during normal activities of daily living. Fibrocartilage composes the intervertebral discs separating the vertebrae of the spinal columns. Elastic cartilage is present in areas requiring extreme resilience, such as the tip of the nose.

[134] The ability of cartilage to rapidly and reversibly change shape is attributable to a resilient and elastic matrix with a high content of highly soluble proteoglycans entrapped within collagen, an insoluble fiber network. Proteoglycans, collagen and other molecules present in the cartilage tissue are produced by mesenchymally-derived cartilage cells, the chondrocytes. Chondrocytes receive nutrients and dispose wastes by diffusion through the matrix and are believed to have limited mobility or ability to divide and regenerate damaged tissue.

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[135] Chondrocytes normally produce anti-angiogenesis factors. However, when large areas of cartilage are damaged, overgrowth by fibroblasts and neovascularization of the area may result in the formation of scar tissue or a callus instead of articular cartilage. A subsequent ingrowth of bone forming cells may result in calcium deposition in these areas, causing further deformation of the local area.

[136] Subchondral bone supports the overlying articular cartilage and transmits load to and from cartilage, and therefore contributes to the structural and functional integrity of the cartilage. Some studies suggest restoration of subchondral bone in an osteochondral defect will create a beneficial mechanical environment for the remodeling of neo-cartilaginous tissue and its integration with the surrounding host cartilage. See Smith, et al., "Analysis of the Mechanical Environment in a Repairing Osteochondral Defect", Trans ORS, 47:442 (2001); Wayne, et al., "A u-p Finite Element Analysis of the Behaviors of a Repaired Cartilage Surface", Trans ORS, 37:75 (1991). However, most of the repair and implant strategies to treat an osteochondral defect to date utilize deformable materials that do not have sufficient osteo-conductivity and mechanical strength, which may compromise the results of the repair.

[137] A variety of materials can be used as cartilage repair matrices, some of which include material obtained from autologous, allogenic, or xenogenic cartilage while others do not. Transfer of cartilage cells from healthy regions of the joint to diseased surfaces in order to restore joint function has also been attempted. In this context, cartilage cells or small regions of cartilage are placed in partial or full-thickness defects within the joint surface using an open surgical procedure. The cell construct is held in place by periosteal tissue that is sutured in place. However, implanting cells or resurfacing with autogenous or allograft cartilage in the absence of an organized extracellular matrix does not support normal weight bearing. In many cases, these grafts quickly become fibrillated and degrade. With any type of cartilage exchange, efficacy of repair will be greatly facilitated following restoration of an extra-cellular matrix structure of normal cartilage prior to use.

[138] Other approaches for repairing cartilage seed cartilage cells on a collagen matrix that is subsequently implanted. For example, U.S. Pat. No. 6,080,194 describes a collagen template formed by combining a porous collagen sponge with a collagen membrane. Other methods involve implantation of cells. U.S. Pat. No. 5,786,217 describes methods and compositions for the ex vivo proliferation of cells and their implantation to repair articular cartilage defects; U.S.

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Pat. No. 5,206,023 discloses methods and compositions for treatment and repair of defects or lesions of the cartilage; and, U.S. Pat. No. 5,041,138 concerns neomorphogenesis of cartilage in vivo from cell culture for the growth and implantation of cartilaginous structures. However, these methods do not provide much physiological support to the implanted cells, and not much access to the natural blood supply, limiting these procedures to applications with respect to the size of the defect being treated and the amount of load bearing possible.

[139] Different approaches have been performed to recruit progenitor cells or chondrocytes in an osteochondral or chondral defect, including penetration of subchondral bone in order to access mesenchymal stem cells (MSCs) in the bone marrow which have the potential to differentiate into cartilage and bone. Steadman, et al., "Microfracture: Surgical Technique and Rehabilitation to Treat Chondral Defects", Clin Orthop., 391 S:362-369 (2001). In addition, some factors in the body are believed to aid in the repair of cartilage. For example, it has been observed that transforming growth factors beta (TGF-b) have the capacity to recruit progenitor cells into a chondral defect from the synovium or elsewhere when TGF-b is loaded in the defect. Hunziker, et al., "Repair of Partial-Thickness Defects in Articular Cartilage: Cell Recruitment From the Synovial Membrane", J. Bone Joint Surg., 78-A:721-733 (1996). However, technical problems associated with the application of growth factors as cartilage repair strategies include the uncertainty of the initial dosage and the timing of release of these extrinsic bioactive factors. Further, the interaction among multiple bioactors (growth factors, cytokines, transcription factors) in natural chondrogenic development is not well understood, which may be a contributing reason to the failure of using a single growth factor for therapeutic purposes.

[140] U.S. Patent Nos. 5,270,300 and 5,041,138 both describe a method for treating defects or lesions in cartilage which provides a matrix, possibly composed of collagen, with pores large enough to allow cell population, and which further contains growth factors or other factors (e.g. angiogenesis factors) appropriate for the type of tissue desired to be regenerated. U.S. Patent Nos. 5,270,300 and 5,041,138 both teach the use of TGF-beta in the matrix as a proliferation and chemotactic agent at a lower concentration, and a subsequent release of the same factor at a higher concentration to induce differentiation of cartilage repair cells.

[141] Alternative methods of treatment use "plugs" of viable cartilage from the edge joint that are implanted into the damaged areas. These have limited success, in that only small defects can be treated, and vascularization of the seeded plug is difficult.

[142] One important deficiency in the prior methods is the lack of a means to induce high levels of cartilage expression in the cartilage cells at the site of implantation, and there is insufficient vascularization and angiogenesis of the implant. Accordingly, most of the proliferative cartilage cells die, resulting in poor repair of the defect.

[143] In accordance with certain embodiments of the present invention, various agents are incorporated into a bone or cartilage matrix such as those described above, resulting in a matrix with improved osteogenic and/or chondrogenic activity. The incorporation of any peptides, protein fragments, proteases, and/or other molecules described herein into the inventive bone and cartilage matrix compositions, is generally accomplished by suspending the molecule or molecules of interest in an appropriately compatible buffer as will be known to those skilled in the art. This buffer may be mixed with lyophilized matrix in a relatively low liquid-to-solid volume ratio to form a slurry. The slurry is then lyophilized and used to prepare the desired formulations. One or more peptides, protein fragments, and/or proteases may also be combined with the bone or cartilage by soaking or immersing the bone or cartilage in a solution or dispersion of the desired bioactive agents. Alternatively or additionally, bioactive agents may be applied to the implant by spraying, dipping, soaking, etc. Any bioactive agent may be adsorbed to the bone or cartilage using such methods well known in the art.

[144] As described herein, many of the osteoinductive or chondrogenic factors found in a bone or cartilage matrix are in cryptic form and must be "activated" or "released" in order to be osteoinductive. The activation of osteoinductive factors may involve a conformational change, a post-translational modification, protein cleavage, a change in tertiary or quaternary structure, release from a binding protein, *etc.* In preferred embodiments, the factors are in a pre- or pro-form, which requires proteolytic cleavage to be active. The osteoinductive factors may also be associated with a binding protein or a protein of a bone or cartilage matrix. Proteolysis may also be involved in the activation or inactivation of a binding protein, which could result in activation of the osteoinductive peptide or protein fragment. Therefore, all treatments of a bone or cartilage matrix with any specific or non-specific condition may affect activation rates of osteoinductive peptides and protein fragments.

[145] According to the present invention, the presence or activation of peptides and/or protein fragments having osteoinductive or chondrogenic activity compensates for the existing degradation of osteoinductive or chondrogenic proteins in the matrix. In certain preferred

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embodiments it is desirable to both inhibit the degradation of osteoinductive or chondrogenic factor and activate or add the osteoinductive or chondrogenic peptides or protein fragments of the invention. As previously mentioned, such factors as pH, ion concentration, or other factors which affect protein function and/or folding of the peptide or protein fragment may affect the activation of osteoinductive or chondrogenic factors found in bone or cartilage matrices. These factors also may affect the release of a factor from its binding protein. For example, where pH plays a role in the activation of a factor, the matrix composition may include a chemical compound such as a polymer which will break down over time and release an acid by-product; thereby, activating the factors within the matrix composition. Alternatively, a biodegradable polymer may release ions or a protease that is able to "activate" the osteoinductive factors of the matrix composition.

[146] As will be appreciated by those of skill in this art, the osteoinductive peptides and protein fragments of the invention have the effect of increasing the overall osteoinductivity or chondrogenic activity of a bone or cartilage matrix. One of skill in the art can appreciate that increasing the osteoinductivity of a bone or cartilage matrix shortens the time for bone regeneration to occur and improves the graft treatment process.

[147] To the improved bone matrices of the present invention may be added other osteoinducing agents. These agents may be added in an activated or non-activated form. These agents may be added at anytime during the preparation of the inventive material. For example, in a demineralized bone matrix, the osteoinducing agent may be added after the demineralization step and prior to the addition of any stabilizing agents. In certain embodiments, the demineralized bone matrix is lyophilized in a solution containing the osteoinducing agent. In other embodiments, the osteoinducing agents are adhered onto a hydrated demineralized bone matrix and are not freely soluble. In other instances, the osteoinducing agent is added to a demineralized bone matrix after addition of any stabilizing agent so that the osteoinducing agent is available immediately upon implantation.

[148] Osteoinducing agents include any agent that leads to or enhances the formation of bone. The osteoinducing agent may do this in any manner, for example, the agent may lead to the recruitment of cells responsible for bone formation, the agent may lead to the secretion of matrix which may subsequently undergo mineralization, the agent may lead to the decreased resorption of bone, *etc.* Particularly preferred osteoinducing agents include bone morphogenic proteins

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(BMPs), transforming growth factor (TGF-β), insulin-like growth factor (IGF-1), and angiogenic factors such as VEGF. In one preferred embodiment (see U.S.S.N. 10/271,140, filed October 15, 2002, incorporated herein by reference), the osteoinducing agent is genetically engineered to comprise an amino acid sequence, which promotes the binding of the inducing agent to the demineralized bone matrix or the carrier. Sebald et al. in PCT/EP00/00637, incorporated herein by reference, describe the production of exemplary engineered growth factors, suitable for use with demineralized bone matrices.

[149] Those skilled in the art will readily appreciate that the same principles can be applied to cartilage repair matrices. Chondrogenic agents include any agent that leads to or enhances the formation of cartilage. The chondrogenic agents may do this in any manner, for example, the agent may lead to the recruitment of cells responsible for cartilage formation, the agent may lead to the secretion of matrix, the agent may lead to the resorption of cartilage.

[150] VI. Formulation

[151] Improved osteogenic and chondrogenic matrix compositions of the present invention may be formulated for a particular use. The formulation may be used to alter the physical, biological, or chemical properties of a bone or cartilage graft preparation. A physician would readily be able to determine the formulation needed for a particular application taking into account such factors as the type of injury, the site of injury, the patient's health, the risk of infection, *etc.*

[152] Inventive compositions therefore may be prepared to have selected osteoinductivity or chondrogenic activity rates, or even to have different rates in different portions of an implant. In certain embodiments, an inventive formulation may include a mixture of active peptides or protein fragments, each with a different half-life. Such a mixture could extend the period of osteoinductivity or chondrogenic activity in the composition. The density distribution and/or type distribution of the peptides can be varied to selectively control properties such as the rate of remodeling and resorption of an implant.

[153] In certain preferred embodiments of the invention, $1cm^3$ of compositions such as this can be formulated to stimulate bone growth in a human patient comparable to the bone growth induced by treatment with 0.1-10 ug of rhBMP-2 (recombinant human BMP-2) on a $1cm^3$ collagen sponge, and preferably comparable to 10-100 ug, and most preferably comparable to 0.1-100 mg rhBMP-2 on such a sponge. The effect on bone growth of these compositions can be

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compared to that of rhBMP-2 or other growth factors in an athymic rat model assay according to the method of Edwards et al. ("Osteoinduction of Human Demineralized Bone: Characterization in a Rat Model" Clinical Orthopeadics& Rel. Res., 357:219-228, December 1998) or using other accepted models.

[154] Physical properties such as deformability and viscosity of the DBM may also be chosen depending on the particular clinical application. Those skilled in the art will appreciate that the particles of the bone matrix or cartilage may be mixed with materials and factors to improve other characteristics of the implant. For example, the improved matrix material may be mixed with other agents to improve wound healing. These agents may include drugs such as antibiotics and/or anti-inflammatory agents, proteins, peptides, polynucleotides, solvents, chemical compounds, and/or biological molecules.

[155] The particles of matrices (or other inventive bone or cartilage material) may also be formed into various shapes and configurations. As mentioned above, the particles can, for example, be formed into rods, strings, sheets, weaves, solids, cones, discs, fibers, wedges *etc.* In certain embodiments, the shape and size of the particles in the bone or cartilage matrix composition affects the time course of osteoinductivity. For example, due to degradation of the bone or cartilage matrix material and diffusion rates of associated factors *in vivo*, with a cone or wedge shape, the tapered end may have osteoinductivity shortly after implantation of the matrix composition, whereas the thicker end may have activity later in the healing process (*e.g.*, hours to days to weeks later). Also, a larger particle size may induce bone formation over a longer time course than smaller particles. Particles of different characteristics (*e.g.*, composition, size, shape) may be used in the formation of these different shapes and configurations. For example, in a sheet of demineralized bone matrix, a layer of long half-life particles may be alternated between layers of shorter half-life particles (See U.S. Patent 5,899,939, incorporated herein by reference). In a weave, strands composed of short half-life particles may be woven together with strands of longer half-lives.

[156] In one preferred embodiment of the invention, fibrous demineralized bone matrix is shaped into a form as described in U.S. Patent 5,507,813 and U.S.S.N. 10/271,140, filed October 15, 2002, incorporated herein by reference. The shaped matrix is then embedded within a diffusion barrier type matrix, such that a portion of the matrix is left exposed free of the matrix material. The matrix is treated as described herein either before or after shaping. Devices

prepared in this way from these matrices have a combination of immediate and longer lasting osteoinductive properties and are particularly useful in promoting bone mass formation in human posterolateral spine fusion indications.

[157] In another embodiment of the invention, demineralized bone matrix compositions have a pre-selected three-dimensional shape prepared by repeated application of individual layers of DBM, for example by 3-D printing as described by Cima et al. U.S. Patents 5,490,962; and 5,518,680, each of which is incorporated herein by reference; and Sachs et al. U.S. Patent 5,807,437, incorporated herein by reference. Different layers may include individual stabilized demineralized bone matrix preparations, or alternatively may include DBM layers treated with stabilizing agents after deposition of multiple layers. The matrix is treated as described herein either before or after shaping.

[158] In the process of preparing improved inventive bone and cartilage matrix materials, the materials may be produced entirely aseptically or be sterilized to eliminate any infectious agents such as HIV, hepatitis B, or hepatitis C. The sterilization may be accomplished using antibiotics, irradiation, chemical sterilization (*e.g.*, ethylene oxide), or thermal sterilization. Other methods known in the art of preparing bone and cartilage matrices, such as defatting, sonication, and lyophilization may also be used in preparing the improved matrix. Since the biological activity of various materials including demineralized bone is known to be detrimentally affected by most terminal sterilization processes, care must be taken when sterilizing the inventive compositions. In preferred embodiments, the matrix compositions described herein will be prepared aseptically or sterilized, see, e.g., U.S.S.N. 10/271,140, filed October 15, 2002.

VII. Testing

[159] Bone formation may be tested in by various methods accepted in the art, for example, in athymic rats using the method of Edwards et al. ("Osteoinduction of Human Demineralized Bone: Characterization in a Rat Model" *Clinical Orthopeadics& Rel. Res.*, 357:219-228, December 1998; incorporated herein by reference). In other instances, osteoinduction is considered to occur through cellular recruitment and induction of the recruited cells to an osteogenic phenotype. Osteoinductivity may also be determined in tissue culture as the ability to induce an osteogenic phenotype in culture cells (primary, secondary, or explants). It is advisable to calibrate the tissue culture method with an *in vivo* ectopic bone formation assay as described

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by Zhang et al. ("A quantitative assessment of osteoinductivity of human demineralized bone matrix" *J. Periodontol.* 68(11):1076-84, November 1997; incorporated herein by reference). Calibration of the *in vitro* assays against a proven *in vivo* ectopic bone formation model is important because the ability of a compound to induce an apparent "osteogenic" phenotype in tissue culture may not always be correlated with the induction of new bone formation *in vivo*. BMP, IGF, TGF-β, and various angiogenic factors are among the osteoinductive factors found to recruit cells from the marrow or perivascular space to the site of injury and then cause the differentiation of these recruited cells down a pathway responsible for bone formation. For example, DBM isolated from either bone or dentin have been found to be osteoinductive materials (Ray et al., "Bone implants" *J. Bone Joint Surgery* 39A:1119, 1957; Urist, "Bone: formation by autoinduction" *Science* 150:893, 1965; each of which is incorporated herein by reference).

[160] Osteoinductivity score refers to a score ranging from 0 to 4 as determined according to the method of Edwards et al. (1998), *supra*, or an equivalent calibrated test. In the method of Edwards et al., a score of "0" represents no new bone formation; "1" represents 1%-25% of implant involved in new bone formation; "2" represents 26-50% of implant involved in new bone formation; "3" represents 51%-75% of implant involved in new bone formation; and "4" represents >75% of implant involved in new bone formation. In most instances, the score is assessed 28 days after implantation. However, the osteoinductivity score may be obtained at earlier time points such as 7, 14, or 21 days following implantation. In these instances it is important to include a normal control such as matrix powder without a carrier, and if possible, a positive control such as BMP. Occasionally osteoinductivity may also be scored at later time points such as 40, 60, or even 100 days following implantation. Percentage of osteoinductivity refers to an osteoinductivity score at a given time point expressed as a percentage of activity, of a specified reference score. Results of tests in animal models can be correlated with effects in human patients, and a comparable osteoinductivity score can be derived. A number of methods by which cartilage repair/growth can be assessed are known in the art. For example, morphological criteria (histology), compressive strength, biochemical composition, and imaging studies (e.g. MRI), have all proven useful in measuring cartilage repair/growth. (See, e.g., Hidaka C, et al., J Orthop Res. 2003 Jul;21(4):573-83; Roberts S Arthritis Res Ther. 2003;5(1):R60-73. Epub 2002 Nov 13, etc., Kavalkovich, K., et al., "Chondrogenic activity of

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mesenchymal stem cells compared to articular chondrocytes", poster presented at the 47th Annual Meeting, Orthopaedic Research Society, Feb. 25-28, San Francisco, CA (published in J. Bone Joint Surgery), Huang, W., et al., Proc. Natl. Acad. Sci., 98(1): 160-165, 2001 for examples.) According to one chondrogenic assay, chondrogenic media with 10ng/ml TGF-ß3, 40 µg/ml proline, 100µg/ml pyruvate and 50 mg/ml ITS (insulin, transferrin and selenious acid) is added to the pellet culture, for a period of time, e.g., 21 days. Chondrocytic phenotype is assessed using safranin-O and H&E stainings and/or by measuring the expression of Type II and/or Type X collagen. The ability of any of the inventive compositions to achieve comparable results may be tested.

[161] In certain embodiments of the invention the improved bone or cartilage matrix composition preferably produce bone or cartilage in an animal model and/or in human patients with similar timing and at a level at least 10%, 20%, 35%, 50%, 100%, 200%, 300%, or 400% or greater osteoinductive or chondrogenic activity than a bone or cartilage matrix lacking the peptides or protein fragments of the invention. Of course, one skilled in the art will appreciate that these values may vary slightly depending on the type of test used to measure the osteoinductivity or chondrogenic activity described above. According to the present invention, the test results may fall within the range of 10% to 35%, 35% to 50%, 50% to 100 %, 100% to 200%, and 200% to 400%. In certain preferred embodiments, when a bone matrix composition is implanted into a bone defect site, such as a fracture, a congenital bone defect, an iatrogenic bone defect, a vertebral fusion, or a site of bone destruction due to underlying disease such as rheumatoid arthritis, the bone matrix composition has an osteoinductivity score of at least 1, 2, 3, or 4 in an animal model and/or in humans.

VIII. Applications

[162] Improved osteogenic and chondrogenic compositions of the present invention may be used to promote the healing of bone and cartilage injuries. The compositions may be used in any bone or cartilage of the body and on any type of injury. For example, specific bones that can be repaired using the inventive material include the ethmoid, frontal, nasal, occipital, parietal, temporal, mandible, maxilla, zygomatic, incus, stapes, malleus, cervical vertebrae, thoracic vertebrae, lumbar vertebrae, sacrum, sternum, ribs, clavicle, scapula, humerus, ulna, radius, carpal bones, metacarpal bones, phalanges, ileum, ischium, pubis, pelvis, femur, patella, tibia,

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fibula, calcaneus, talus, and metatarsal bones. Cartilage at any location within the body can be repaired, including both articular and non-articular cartilage. For example, cartilage in joints such as the knee, shoulder, hip, etc., can be repaired as can cartilage within the nose, in the spine, etc. The type of injury amenable to treatment with the improved matrices include bone or cartilage defects resulting from injury, brought about during the course of surgery, infection, malignancy, or developmental malformation. The inventive material may be useful in orthopaedic, neurosurgical, cosmetic, and oral and maxillofacial surgical procedures such as the repair of simple and compound fractures and non-unions, external and internal fixations, joint reconstructions such as arthrodesis, general arthroplasty, cup arthroplasty of the hip, femoral and humeral head replacement, femoral head surface replacement and total joint replacement, repairs of the vertebral column including spinal fusion and internal fixation, tumor surgery (*e.g.*, deficit filling), discectomy, laminectomy, excision of spinal cord tumors, anterior cervical and thoracic operations, repair of spinal injuries, scoliosis, lordosis and kyphosis treatments, intermaxillary fixation of fractures, mentoplasty, temporomandibular joint replacement, alveolar ridge augmentation and reconstruction, inlay bone grafts, implant placement and revision, sinus lifts, *etc.*

[163] In related embodiments, the compositions of the invention are particularly preferred for delivering osteoinductive or chondrogenic growth factors to the site of the bone or cartilage injury. Other preferred agents to be included in the bone or cartilage matrix for delivery include factors or agents that promote wound healing. However, inventive compositions may alternatively or additionally be used to deliver other pharmaceutical agents including antibiotics, anti-neoplastic agents, growth factors, hematopoietic factors, nutrients, *etc.* Bioactive agents that can be delivered using the inventive bone or cartilage matrix composition include non-collagenous proteins such as osteopontin, osteonectin, bone sialo proteins, fibronectin, laminin, fibrinogen, vitronectin, thrombospondin, proteoglycans, decorin, proteoglycans, beta-glycan, biglycan, aggrecan, veriscan, tenascin, matrix GLA protein hyaluronan; cells; amino acids; peptides; inorganic elements; inorganic compounds; organometallic compounds; cofactors for protein synthesis; cofactors for enzymes; vitamins; hormones; soluble and insoluble components of the immune system; soluble and insoluble receptors including truncated forms; soluble, insoluble, and cell surface bound ligands including truncated forms; chemokines, interleukins; antigens; bioactive compounds that are endocytosed; tissue or tissue fragments; endocrine tissue;

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enzymes such as collagenase, peptidases, oxidases, *etc.*; polymeric cell scaffolds with parenchymal cells; angiogenic drugs, polymeric carriers containing bioactive agents; encapsulated bioactive agents; bioactive agents in time-release form; collagen lattices; antigenic agents; cytoskeletal agents; cartilage fragments; living cells such as chondrocytes, osteoblasts, osteoclasts, fibroblasts, bone marrow cells, mesenchymal stem cells, *etc.*; tissue transplants; bioadhesives; bone morphogenic proteins (BMPs), transforming growth factor (TGF-β), insulin-like growth factor (IGF-1, IGF-2), platelet derived growth factor (PDGF); fibroblast growth factors (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), growth factor binding proteins, *e.g.*, insulin-like growth factor binding protein (IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6); angiogenic agents; anticoagulants, bone promoters; cytokines; interleukins; genetic material; genes encoding bone promoting action; cells containing genes encoding bone promoting action; cells genetically altered by the hand of man; externally expanded autograft or xenograft cells; growth hormones such as somatotropin; bone digestors; antitumor agents; fibronectin; cellular attractants and attachment agents; immunosuppressants; bone resorption inhibitors and stimulators; mitogenic factors; bioactive factors that inhibit and stimulate second messenger molecules; cell adhesion molecules, *e.g.*, cell-matrix and cell-cell adhesion molecules; secondary messengers; monoclonal antibodies specific to cell surface determinants on mesenchymal stem cells; portions of monoclonal antibodies specific to cell surface determinants on mesenchymal stem cells; clotting factors; polynucleotides; and combinations thereof. The amount of the bioactive agent included with the bone or cartilage matrix composition can vary widely and will depend on such factors as the agent being delivered, the site of administration, the patient's physiological condition, *etc.* The optimum levels being determined in a specific case based upon the intended use of the implant.

[164] For example, inventive bone or cartilage matrix compositions may be prepared so that they include one or more compounds selected from the group consisting of drugs that act at synaptic and neuroeffector junctional sites (*e.g.*, acetylcholine, methacholine, pilocarpine, atropine, scopolamine, physostigmine, succinylcholine, epinephrine, norepinephrine, dopamine, dobutamine, isoproterenol, albuterol, propranolol, serotonin); drugs that act on the central nervous system (*e.g.*, clonazepam, diazepam, lorazepam, , benzocaine, bupivacaine, lidocaine, tetracaine, ropivacaine, amitriptyline, fluoxetine, paroxetine, valproic acid, carbamazepine, bromocriptine, morphine, fentanyl, naltrexone, naloxone,); drugs that modulate inflammatory

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responses (*e.g.*, aspirin, indomethacin, ibuprofen, naproxen, steroids, cromolyn sodium, theophylline); drugs that affect renal and/or cardiovascular function (e.g., furosemide, thiazide, amiloride, spironolactone, captopril, enalapril, lisinopril, diltiazem, nifedipine, verapamil, digoxin, isordil, dobutamine, lidocaine, quinidine, adenosine, digitalis, mevastatin, lovastatin, simvastatin, mevalonate); drugs that affect gastrointestinal function (*e.g.*, omeprazole, sucralfate); antibiotics (*e.g.*, tetracycline, clindamycin, amphotericin B, quinine, methicillin, vancomycin, penicillin G, amoxicillin, gentamicin, erythromycin, ciprofloxacin, doxycycline, acyclovir, zidovudine (AZT), ddC, ddI, ribavirin, cefaclor, cephalexin, streptomycin, gentamicin, tobramycin, chloramphenicol, isoniazid, fluconazole, amantadine, interferon,); anti-cancer agents (*e.g.*, cyclophosphamide, methotrexate, fluorouracil, cytarabine, mercaptopurine, vinblastine, vincristine, doxorubicin, bleomycin, mitomycin C, hydroxyurea, prednisone, tamoxifen, cisplatin, decarbazine); immunomodulatory agents (*e.g.*, interleukins, interferons, GM-CSF, TNFα, TNFβ, cyclosporine, FK506, azathioprine, steroids); drugs acting on the blood and/or the blood-forming organs (*e.g.*, interleukins, G-CSF, GM-CSF, erythropoietin, vitamins, iron, copper, vitamin B_{12}, folic acid, heparin, warfarin, coumarin); hormones (*e.g.*, growth hormone (GH), prolactin, luteinizing hormone, TSH, ACTH, insulin, FSH, CG, somatostatin, estrogens, androgens, progesterone, gonadotropin-releasing hormone (GnRH), thyroxine, triiodothyronine); hormone antagonists; agents affecting calcification and bone turnover (*e.g.*, calcium, phosphate, parathyroid hormone (PTH), vitamin D, bisphosphonates, calcitonin, fluoride), vitamins (*e.g.*, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, biotin, choline, inositol, carnitine, vitamin C, vitamin A, vitamin E, vitamin K), gene therapy agents (*e.g.*, viral vectors, nucleic-acid-bearing liposomes, DNA-protein conjugates, anti-sense agents); or other agents such as targeting agents *etc.*

[165] In certain embodiments, the agent to be delivered is adsorbed to or otherwise associated with the matrix being implanted. The agent may be associated with the matrix of the bone or cartilage matrix composition through specific or non-specific interactions; or covalent or non-covalent interactions. Examples of specific interactions include those between a ligand and a receptor, a epitope and an antibody, *etc.* Examples of non-specific interactions include hydrophobic interactions, electrostatic interactions, magnetic interactions, dipole interactions, van der Waals interactions, hydrogen bonding, *etc.* In certain embodiments, the agent is attached to the matrix using a linker so that the agent is free to associate with its receptor or site of action

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in vivo. In other preferred embodiments the agent is either covalently or non-covalently attached to the matrix. In certain preferred embodiments, the agent to be delivered may be attached to a chemical compound such as a peptide that is recognized by the matrix of the bone or cartilage matrix composition. In another embodiment, the agent to be delivered is attached to an antibody, or fragment thereof, that recognizes an epitope found within the matrix of the bone or cartilage matrix composition. In certain embodiments at least two bioactive agents are attached to the bone or cartilage matrix composition. In other embodiments at least three bioactive agents are attached to the bone or cartilage matrix composition.

[166] These and other aspects of the present invention will be further appreciated upon consideration of the following Examples, which are intended to illustrate certain particular embodiments of the invention but are not intended to limit its scope, as defined by the claims. All references cited herein are hereby incorporated by reference.

EXAMPLES

Example 1: The Effect of Furin on Demineralized Bone Matrix

This example relates to the study of the effect of furin and other PPC's on the osteoinductive capacity of human demineralized bone matrix.

A solution of 100 mM HEPES containing 0.5% Triton X-100, 1mM $CaCl_2$, pH 7.5 is prepared. Various amounts of human DBM (e.g., 40 mg) are incubated in the aforementioned HEPES buffer containing furin at concentrations of 0, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 units per ml at temperatures ranging from 4°C to 60°C (e.g., 23°C, 37°C) for periods ranging from 1 hour to 1 week (e.g., 24 hrs).

The above steps are repeated with the addition of 3 mM iodoacetic and/or 0.1mM Benzamidine HCl in order to protect osteoinductivity of DBM. As a control, the experiment is repeated including 1mM EGTA or the specific furin inhibitor $C_{34}H_{66}N_{11}O_5CI$ (1mM) in the HEPES Buffer. At the end of the incubation period the bone is washed with deionized H_2O and lyophilized. 40mg doses are implanted in the quadriceps of nude rats. The animals are euthanized after 28 days and the amounts of bone formation by furin treated DBM and control groups histologically and radiographically quantified (see, e.g., Kawai and Urist, *Clin. Orthop.* (1998) 233:262-267).

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Example 2: Preparing Demineralized Bone Matrix (DBM).

DBM may be prepared using any method or technique known in the art (see Russell et al. *Orthopedics* 22(5):524-531, May 1999; incorporated herein by reference). The following is an exemplary procedure for preparing demineralized bone derived from Glowacki et al. "Demineralized Bone Implants" *Clinics in Plastic Surgery* 12(2):233-241, April 1985, which is incorporated herein by reference. Bones or bone fragments from donors are cleaned to remove any adherent periosteum, muscle, connective tissue, tendons, ligaments, and cartilage. Cancellous bone may be separated from dense cortical bone and processed as large pieces. Cortical bone may be cut into small pieces to improve the efficiency of subsequent washes and extractions. Denser bone from larger animals may need to be frozen and hammered in order to produce chips less than 1 cm. The resulting pieces of bone are thoroughly washed with cold, deionized water to remove marrow and soft tissue.

The cleaned bone is then extracted with frequent changes of absolute ethanol for at least 1 hour. Typically, a total of 4 liters of ethanol is used per 100 g of bone. The bone is then extracted with frequent changes of anhydrous diethyl ether in a fume hood for 1 hour. Typically, 2 liters of ether is used per 100 g of bone. The bone is dehydrated by these extractions of ethanol and ether and can be stored at room temperature.

The dehydrated bone is then frozen and then pulverized in a liquid nitrogen-impacting mill. Pulverized bone is then sieved into fractions of 75 to 250, 250 to 450, and greater than 450 microns. Bone particle fractions are then demineralized using 0.5 M hydrochloric acid (50 ml per gram) for 3 hours at room temperature or at 4°C on magnetic stirrers with insulation to prevent overheating. Large chips of bone and blocks are extracted completely at 4°C with frequent changes of 0.5 M hydrochloric acid. The demineralization process can be monitored radiographically, by ashing, or by nondecalcified histologic techniques (von Kossa stain). The acid and liberated minerals are washed away with cold, deionized water until the pH of the wash matches the pH of the water. The water washes can be decanted from the large particles and

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chips of bone; however, the washes must be removed by centrifugation from the finer particles. The washing step requires approximately 500 ml of water per gram of starting bone particles.

Demineralized bone powders are extracted with changes of absolute ethanol for 1 hour using 200 ml of ethanol per gram of starting bone particles. The material is extracted in a fume hood with changes of anhydrous ethyl ether for 1 hour with 100 ml of ether per gram of starting bone particles. After the last change of ether is removed, the demineralized bone powder is left overnight in the hood until all the residual ether has vaporized. The particles should be odorless, snow-white, and discrete. To sterilize the demineralized bone material, it may be treated with cold ethylene oxide or irradiated.

To test the bioactivity of the prepared DBM, 25 mg of the material is implanted into each of two thoracic subcutaneous pockets in shaved, anesthetized 28-day old male Charles River CD rats. The implanted specimens may then be harvested and inspected several days after implantation. The composition of the induced tissue can be quantified by histomorphometric analysis and be biochemical techniques.

Example 3: Another Method of Preparing DBM.

DBM may be prepared using any method or techniques known in the art (See Russell et al., *Orthopedics* 22(5):524-531, May 1999; incorporated herein by reference).

Demineralized bone matrix is prepared from long bones. The diaphyseal region is cleaned of any adhering soft tissue and then ground in a mill. Ground material is sieved to yield a powder with particles approximately 100 μm to 500 μm in diameter. The particulate bone is demineralized to less than about 1% (by weight) residual calcium using a solution of Triton X-100 (Sigma Chemical Company, St Louis, MO) and 0.6N HCl at room temperature followed by a solution of fresh 0.6N HCl. The powder material is rinsed with deionized water until the pH was greater than 4.0. It then is soaked in 70% ethanol and freeze-dried to less than 5% residual moisture.

Example 4: Determining Time Course for Induction of Bone Growth by Intermuscular Implant

[167] This Example characterizes the time course of induction of bone growth in an intermuscular site using the inventive materials, as compared with DBM base powder (as in Example 1), at time points of 7, 14, 28, and 35 days. This Example is adapted from the rat

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model for assessing osteoinduction of DBM found in Edwards et al. "Osteoinduction of Human Demineralized Bone: Characterization in a Rat Model" *Clinical Orthopaedics* 357:219-228, December 1998; incorporated herein by reference.

[168] The study is conducted in athymic (nude) rats in order to minimize the potential for a cross-species incompatibility response to human tissue implants. The hind-limb intermuscular site is used for the initial determination of heterotopic bone induction properties because the site does not naturally contain bone.

[169] Rats, for example, female homozygous rnu/rnu rats in the 50-75 g range are obtained. The rats are housed for one week for acclimatization purposes prior to surgery. Sterile microisolator cages are used throughout the investigation, with sterile water and rodent diet provided *ad libitum*.

[170] *Implant Placement*: A single intermuscular (IM) site is utilized in each hind limb of 30 rats. To provide a common positive control over all animals, a single 40 mg sample of rat DBM powder is placed intramuscularly within the left pectoralis (LP) muscle of each rat. Animals are allowed normal activities following surgical procedures.

[171] *Implant Materials*: DBM and test materials are kept at room temperature. Eight 145 mg samples of Test and eight 40-mg samples of DBM powder are tested for implantation times of 7, 14, and 28 days. Six samples of each are tested at 35 days. The 40 mg samples of DBM powder are rehydrated with 100 µl of sterile ALLOPREP™ (Ostetotech, Eatontown, NJ). Each of the samples is packed into a 1 ml blunt cut syringe. Implantation is randomized so that a single animal does not receive two of the same implants.

[172] *Anesthesia*: The rats are anesthetized with a mixture of ketamine (200 mg), xylazine (400 mg), and physiological saline (10 ml). The dosage was 3.5 ml/kg body weight administered intraperitoneally.

[173] *Procedure:* Aseptic surgical procedures are carried out in a laminar airflow hood. A 1-cm skin incision is made on each upper hind limb using a lateral approach, and the skin is separated from the muscle by blunt dissection. A superficial incision aligned with the muscle plane is made to allow for insertion of the tips of the scissors. Blunt dissection is performed from this line deep into the muscle to create a pocket to hold the implanted material. A single suture is inserted to close the muscle pocket, and the skin is closed with metal clips.

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[174] Implantation of specimens in the left pectoralis muscles involved making a 1-cm skin incision over the chest, blunt dissection of the muscle to create a pocket, and positioning of the rat DBM powder using a blunt syringe. A single suture is inserted to close the muscle pocket, and the skin is closed with metal clips.

[175] Rats are euthanized with CO_2 following the designated implantation time. Implant materials are located by palpitation, retrieved by blunt dissection, and cleaned of the surrounding tissue by careful trimming. An observer blinded to implant type performed a macroscopic evaluation of the implant material. Color, vascularity, hardness, and integrity are scored according to the scheme outlined in the Table below. (The highest score for the most robust response would be a 4 while a specimen showing little or no osteoinductive potential would score a 0.) Experience with this model has shown a high correlation between visual observations and histological observations of implant performance only at the extremes of both ends of the scale.

Macroscopic Observation Scoring Guidelines

Color:	White (W)	Grey (G)	Red (R)
Vascularity:	None (N)	Some (S)	Robust (R)
Hardness:	Mushy (M)	Firm (F)	Hard (H)
Integrity:	Diffuse (D)	Flat (F)	Nodule (N)
Score:	0	0.5	1

[176] *Histology:* Retrieved materials are fixed in Neutral buffered formalin. After fixation in formalin, samples are decalcified in 10% formic acid, dehydrated in graded alcohols, embedded in JB-4 (glycol methacrylate, Polysciences, Inc., Warrington, PA) and sectioned. Five-micron sections are stained with toluidine blue and evaluated by light microscopy.

[177] These explants are histologically evaluated using a semiquantitative method. Briefly, a numerical score based on a five-point scale is assigned to each section of nodule: 4 = more than 75% involved in new bone formation; 3 = 51-75% involved in new bone formation; 2 = 26-50% involved in new bone formation; 1 = 1-25% of the explant involved in new bone formation; and 0 = no evidence for the process of endochondral bone formation including the presence of

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cartilage or chondrocytes, active osteoblasts, osteoid, newly formed and mineralized bone, and/or marrow and associated fat cells.

Scoring of Histological Sections

Score	New Bone Formation
0	No new bone formation
1	<25% new bone formation
2	26-50% new bone formation
3	51-75% new bone formation
4	>75% new bone formation

[178] Following histological analysis, average scores are calculated for each material type. Based on previous experience with this animal model, each group is assigned an assessment of osteoinductive potential based on the average histological score.

[179] Example 5: Evaluating Efficacy of Inventive Compositions in Healing Bone Defects *Background Information:* Morselized autogenous cancellous bone (ABG) has long been considered the "gold standard" for osteoinduction when a bone graft is required in an orthopedic clinical situation. Unfortunately, the amount of ABG available is limited, and there is at least a 5% surgical morbidity associated with the harvesting procedure. Demineralized bone matrix (DBM) has been shown to have equal to superior healing potential to ABG.

[180] The rabbit ulna defect model has been modified and used in numerous projects to test the efficacy of osteoinductive and osteoconductive growth factors and matrices as substitute to autogenous bone graft. This study can evaluate the bone inducing capacity of the new DBM formulation grafting material in comparison to previous formulations and ABG.

[181] *Materials and Methods:*

Study Design Summary:

A. Rabbit bilateral 2-cm ulnar defects.

Treatment groups:

1. DBM + osteoinductive peptides or protein fragments
2. DBM + protease

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3. DBM alone

4. Autograft (historical data used for comparison)

Surgical Procedure: Six months old male New Zealand white rabbits are used. A 2.0 centimeter non-uniting defect is surgically created in the bilateral ulnae of all rabbits. After complete periostectomy, thorough defect wash, and partial diaphyseal wash, grafting is implanted (according to test groups) via open surgical technique into each defect. The wound is closed primarily in layers. When anesthesia is achieved, both forelimbs are shaved and prepared with the rabbit supine (limbs up) position. Longitudinal incisions (3-4 cm) are made over both ulnae and the diaphysis (midshaft) portion of the ulna is exposed. The distal osteotomy is made 1 cm from the ulnocarpal (wrist) joint and the proximal osteotomy made 3.0cm from the ulnocarpal joint, to create a 2 cm defect. The osteotomies are created with a high speed burr. The resultant loose block of diaphyseal bone is excised with its periosteum intact. Due to the very adherent interosseous membrane of the rabbit forelimb, internal fixation may not be required. After irrigation with sterile saline to remove blood, bone, and marrow remnants, the implant material is placed in the defect. The deep fascial layer is closed as an envelope around the defect with 3-0 chromic suture. The skin is closed with interrupted nylon suture. A post-operative dressing/splint is applied and removed on the fourth post-operative day.

Radiographs: Antero-posterior radiographs may be obtained immediately post-operatively and additional radiographs are taken at 3, 6, 9, and 12 weeks. High resolution (Faxitron) radiographs may be taken of both limbs after excision and cleaned of soft tissue at either 6 or 12 weeks. Three blinded observers asses each time point for bone formation and remodeling.

Example 6: Osteoinduction in a rabbit model

Introduction and methods: Fifty-five male New Zealand White rabbits are assigned to three treatment groups. Test article is first prepared (e.g., DBM with a protease or with peptides and protein fragments having osteoinductivity). Those animals assigned to the Low Dose treatment group (n=20) receive 3.5 ml of the test article in the right paravertebral muscle following a protocol specified procedure. Animals assigned to the High Dose treatment group (n=20) receive 3.5 ml of the test article in the right paravertebral muscle and 7.0 ml of the test article in the subcutaneous tissue of each side of the dorsal thoracic area. Some animals are assigned to the Control treatment group and are implanted with 3.5 ml of control article

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(rehydrated DBM powder) in the right paravertebral muscle. At 7, 14, and 28 days post-implantation, animals from the Low and High Dose treatment groups and animals from the Control groups are humanely sacrificed. At 60 days post-implantation, the remaining animals are sacrificed. The implant sites are collected from each rabbit and fixed in 10% neutral buffered formalin (NBF). The test and control implant sites from the 60 days post-implantation study interval are placed in decalcification solutions for 3 days after adequate formalin fixation. All tissue samples are processed using standard histological techniques, sectioned at 5 μm, and stained with hematoxylin and eosin.

Example 7: Terminal Sterilization

This example describes a terminal sterilization method, which minimizes osteoinductivity loss in the inventive preparations.

The inventive DBM preparations are produced in a clean room environment from human tissue. The finished implants are placed in individual tray packages.

Each tray is placed in an Audionvac sealing apparatus (Audion Electro B.V., Weesp-Holland), which is supplied with a cylinder consisting of 50/50 hydrogen/argon gas. Before the tray packages are sealed, they are evacuated and backfilled with the gas mixture twice. Following sealing, the gas mixture remains in each tray package.

The packaged implants are then sealed packages and then treated with 15 KGy gamma radiation from a cobalt 60 source to reduce the bioburden of the implants to the desired level

Example 8: Process of making a species-specific osteoimplant with defined dimensions.

Long bones from human Rhesus Monkey, canine, and rabbit are used to prepare species-specific solid formed implant matrices. Bones are aseptically cleaned. The cortical bone is processed in the bone milling apparatus described in U.S. Patent No. 5,607,269, incorporated herein by reference, to yield about 65 grams of elongate bone fibers. The elongate bone fibers are placed in a reactor and allowed to soak for about 5-10 minutes in 0.6 N HCl plus 20-2000 ppm nonionic surfactant solution. Following drainage of the HCl/surfactant, 0.6 N HCl at 15 ml per gram of total bone is introduced into the reactor along with the elongate bone fibers. The reaction proceeds for about 40-50 minutes. Following drainage through a sieve, the resulting demineralized elongate bone fibers are rinsed three times with sterile, deionized water at 15 ml

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per gram of total bone, being replaced at 15-minute intervals. Following drainage of the water, the bone fibers are covered in alcohol and allowed to soak for at least 30 minutes. The alcohol is then drained and the bone fibers are rinsed with sterile, deionized water. The bone fibers are then contacted with a mixture of about 4.5 ml glycerol per gram of dry bone fibers and about 10.5 ml sterile deionized water per gram of dry bone fibers s for at least 60 minutes. Excess liquid is drained and the resulting liquid composition containing approximately 11% (w/v) demineralized, elongate bone fibers was transferred to a 11cm x 11cm mold containing a lid having a plurality of protruding indentations (approximately 1.5cm x 3.5cm in width and length, and 4mm in depth), the lid is gently placed on the mold such that the indentations become immersed into the fibers to exert as little pressure on the composition as possible. The dimensions of the protrusions can be made specific for the size of the osteoimplant required for the animal model of interest. The resulting cut pieces have specified dimensions of, e.g., 4.5 cm in length, 2.5 cm in width and about 8 mm in height (or thickness) with trough dimensions 3.5 cm in length, 1 cm in width and depth of the of 4 mm. The mold is then placed in an oven at 46°C for 4 hours. The composition is then frozen overnight at -70°C and then lyophilized for 48 hours. Following lyophilization, the mold is disassembled and the sponge-like formed composition is cut into individual pieces that contained troughs.

The resulting composition is cohesive, flexible, sponge-like with an obvious continuous three-dimensional structure with visible open pores, has a defined shape including the indentations made by the lid protrusions, does not require rehydration before use, but is rapidly hydratable and retained its shape once wetted with fluids and freezing is not required for storage.

Example 9: Osteoinduction of DBM composition in an Athymic Rat Model

The purpose of this Example is to evaluate the osteoinductive potential of DBM compositions using a heterotopic osteoinductive 28-day implant model (Edwards *et al.*, *Clin. Orthop. Rel. Res.* 357:219-228, 1998; Urist, *Science* 150:893-899, 1965; each of which is incorporated by reference). The DBM composition includes cuboidal shaped DBM particles in combination with DBM fibers (See USSN 60/159,774, filed October 15, 1999; WO0232348; each of which is included herein by reference). Chondrocytes are the predominant cell type in the cube of the DBM following 28-day implantation. This study extends the implant time to 49 days to look evidence of continued bone remodeling within the demineralized cortical cube.

Materials and Methods: Equal volumes of crunch samples weighing approximately 600 mg are packaged in 2.5 ml blunt tipped syringes. Eighteen female athymic rats are obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Animals' weights at the time of surgery are measured. 28-day and 49-day implants are evaluated.

The implant sites are assessed histologically. The fiber component is scored independently of the cubes and is assigned a numerical score based on a 5 point semiquantitative scale based on percent of fiber area involved in new bone formation. The cube portion is assigned a score based on the percent of central Haversian systems involved in new bone formation.

Other Embodiments

The foregoing has been a description of certain non-limiting preferred embodiments of the invention. Those of ordinary skill in the art will appreciate that various changes and modifications to this description may be made without departing from the spirit or scope of the present invention, as defined in the following claims.

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Claims

1. A method of increasing the osteoinductivity of a bone matrix, the method comprising the steps of:

providing a bone matrix; and

contacting the bone matrix with at least one protease that cleaves at least one protein to generate osteoinductive peptides or protein fragments, wherein the peptides or protein fragments cause an increase in osteoinductivity of the bone matrix contacted with the protease resulting in improved bone formation compared to a bone matrix not contacted with the protease.

2. A method of increasing the osteoinductivity of a bone matrix, the method comprising the steps of:

providing a bone matrix; and

contacting the bone matrix with a chemical or condition that generates active osteoinductive peptides or protein fragments, wherein the peptides or protein fragments cause an increase in osteoinductivity of the bone matrix contacted with the protease resulting in improved bone formation compared to a bone matrix not contacted with the protease.

3. The method of claim 1 or 2, wherein the bone matrix comprises mineralized bone matrix, partially demineralized bone matrix, demineralized bone matrix, deorganified bone matrix, anorganic matrix, or a mixture thereof.

4. The method of claim 1 or 2, wherein the peptides or protein fragments are derived from a growth factor.

5. The method of claim 4, wherein the growth factor is selected from the group consisting osteogenic factors, vascularizing factors, macrophage colony stimulating factor (MCSF), insulin-like growth factors (IGF), angiogenic factors, osteonectin, alpha-2-HS glycoprotein, transforming growth factor (TGF), and bone morphogenic protein (BMP).

6. The method of claim 5, wherein the BMP is selected from the group consisting of BMP2, BMP3, BMP5, BMP6, BMP7, BMP8, BMP9, BMP10, BMP11, BMP12, or BMP13.

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7. The method of claim 1 or 2, wherein the active osteoinductive peptides or protein fragments are derived from non-collagenous proteins.

8. The method of claim 1, wherein the active osteoinductive peptides or protein fragments are generated by the protease by cleaving an inactive protein to generate active peptides or protein fragments.

9. The method of claim 8, wherein the inactive protein is a proprotein.

10. The method of claim 9, wherein the proprotein is a TGF superfamily proprotein.

11. The method of claim 10, wherein the TGF superfamily proprotein is proTGF-β.

12. The method of claim 9, wherein the proprotein is a proBMP protein.

13. The method of claim 12, wherein the proBMP protein is proBMP-4 or proBMP-2.

14. The method of claim 9, wherein the protease is a proprotein convertase protease.

15. The method of claim 14, wherein the proprotein convertase protease is furin.

16. The method of claim 1 or 2, wherein the peptides or protein fragments are modified.

17. The method of claim 1 or 2, wherein the peptides or protein fragments are covalently or non-covalently attached to the matrix.

18. The method of claim 1 or 2, further comprising contacting the bone matrix with peptides or protein fragments that are capable of enhancing the osteoinductivity of the bone matrix resulting in improved bone formation ability as compared to the composition without the peptides or protein fragments.

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19. The method of claim 1 or 2, wherein the matrix further comprises multiple growth factors having osteogenic activity.

20. The method of claim 1 or 2, wherein the matrix further comprises one or more hormones.

21. The method of claim 20, wherein the hormone is selected from the group consisting of estrogen, prostaglandin, or parathyroid hormone.

22. The method of claim 1 or 2, wherein the matrix further comprises one or more bioactive agents.

23. The method of claim 22, wherein the bioactive agent is selected from the group consisting of small molecules, chemical compounds, cells, polynucleotides, proteins, peptides, drugs, and viruses.

24. The method of claim 22, wherein the bioactive agent is selected from the group consisting of antibiotics, anti-neoplastic agents, growth factors, hematopoietic factors, wound healing factors, and nutrients.

25. The method of claim 1 or 2, wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 10% or greater.

26. The method of claim 1 or 2, wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 20% or greater.

27. The method of claim 1 or 2, wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 35% or greater.

28. The method of claim 1 or 2, wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 50% or greater.

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29. The method of claim 1 or 2, wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 100% or greater.

30. The method of claim 1 or 2, wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 200% or greater.

31. A method of increasing the chondrogenic activity of a cartilage repair matrix, the method comprising steps of:

providing a cartilage repair matrix; and

contacting the cartilage repair matrix with at least one protease that cleaves at least one protein to generate chondrogenic peptides or protein fragments, wherein the peptides or protein fragments cause an increase in chondrogenic activity of the cartilage repair matrix contacted with the protease resulting in improved cartilage formation compared to a cartilage repair matrix not contacted with the protease.

32. A method of increasing the chondrogenic activity of a cartilage repair matrix, the method comprising steps of:

providing a cartilage repair matrix; and

contacting the cartilage repair matrix with a chemical or condition that generates active chondrogenic peptides or protein fragments, wherein peptides or protein fragments cause an increase in chondrogenic activity of the cartilage repair matrix contacted with the protease resulting in improved cartilage formation compared to a cartilage repair matrix not contacted with the protease.

33. The method of claim 31 or 32, wherein the peptides or protein fragments are derived from a growth factor.

34. The method of claim 33, wherein the growth factor is selected from the group consisting osteogenic factors, vascularizing factors, macrophage colony stimulating factor (MCSF), insulin-

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like growth factors (IGF), angiogenic factors, osteonectin, alpha-2-HS glycoprotein, transforming growth factor (TGF), and bone morphogenic protein (BMP).

35. The method of claim 34, wherein the BMP is selected from the group consisting of BMP2, BMP3, BMP5, BMP6, BMP7, BMP8, BMP9, BMP10, BMP11, BMP12, or BMP13.

36. The method of claim 31 or 32, wherein the peptides or protein fragments are derived from non-collagenous proteins.

37. The method of claim 31, wherein the protease cleaves an inactive protein to generate active peptides or protein fragments.

38. The method of claim 37, wherein the inactive protein is a proprotein.

39. The method of claim 38, wherein the proprotein is a TGF superfamily proprotein.

40. The method of claim 39, wherein the TGF superfamily proprotein is proTGF-β.

41. The method of claim 38, wherein the proprotein is a proBMP protein.

42. The method of claim 41, wherein the proBMP protein is proBMP-4 or proBMP-2.

43. The method of any one of claims 38, wherein the protease is a proprotein convertase protease.

44. The method of claim 43, wherein the proprotein convertase protease is furin.

45. The method of claim 31 or 32, wherein the peptides or protein fragments are modified.

46. The method of claim 31 or 32, wherein the peptides or protein fragments are covalently or non-covalently attached to the cartilage repair matrix.

47. The method of claim 31 or 32, further comprising contacting the cartilage repair matrix with peptides or protein fragments that are capable of enhancing the chondrogenic activity of the cartilage repair matrix resulting in improved cartilage formation ability compared to the composition without the peptides or protein fragments.

48. The method of claim 31 or 32, wherein the cartilage repair matrix further comprises multiple growth factors having chondrogenic activity.

49. The method of claim 31 or 32, wherein the cartilage repair matrix further comprises at least one hormone.

50. The method of claim 49, wherein the hormone is selected from the group consisting of estrogen, prostaglandin E2, and parathyroid hormone (PTH).

51. The method of claim 31 or 32, wherein the cartilage repair matrix further comprises one or more bioactive agents.

52. The method of claim 51, wherein the bioactive agent is selected from the group consisting of small molecules, chemical compounds, cells, polynucleotides, proteins, peptides, drugs, and viruses.

53. The method of claim 51, wherein the bioactive agent is selected from the group consisting of antibiotics, anti-neoplastic agents, growth factors, hematopoietic factors, wound healing factors, and nutrients.

54. The method of claim 31 or 32, wherein the cartilage repair matrix further comprises one or more anti-inflammatory or pro-inflammatory modulators or drugs.

55. The method of claim 31 or 32, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 10% or greater.

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56. The method of claim 31 or 32, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 20% or greater.

57. The method of claim 31 or 32, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 35% or greater.

58. The method of claim 31 or 32, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 50% or greater.

59. The method of claim 31 or 32, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 100% or greater.

60. The method of claim 31 or 32, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 200% or greater.

61. A method of increasing the osteoinductivity of a bone matrix comprising steps of:

contacting a bone matrix with at least one protease that selectively degrades inhibitors of osteogenic activity, wherein the bone matrix has increased osteogenic activity resulting in improved bone formation compared to a bone matrix not contacted with the protease.

62. The method of claim 61, wherein the bone matrix is mineralized, partially demineralized, demineralized, deorganified, anorganic, or a mixture thereof.

63. The method of claim 61, wherein the inhibitor of osteogenic activity is noggin.

64. The method of claim 61, wherein the inhibitor of osteogenic activity is chrondin.

65. The method of claim 61, wherein the inhibitor of osteogenic activity is follistatin.

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66. A method of increasing the chondrogenic activity of a cartilage repair matrix comprising steps of:

contacting a cartilage repair matrix with at least one protease that selectively degrades inhibitors of chondrogenic activity, wherein the cartilage repair matrix has increased chondrogenic activity resulting in improved cartilage formation compared to a cartilage repair matrix not contacted with the protease.

67. An osteoinductive bone matrix composition for implantation at a bone defect site which comprises a bone matrix treated with at least one protease, wherein the protease causes cleavage of inactive proteins to generate osteoinductive peptides or protein fragments, wherein the osteoinductivity of the treated matrix compared to an untreated matrix is increased resulting in improved bone formation.

68. An osteoinductive bone matrix composition for implantation at a bone defect site which comprises a bone matrix treated with a chemical or condition, wherein the chemical or condition generates active osteoinductive peptides or protein fragments, wherein the osteoinductivity of the treated matrix compared to an untreated matrix is increased resulting in improved bone formation.

69. The composition of claim 67 or 68, wherein the bone matrix comprises mineralized bone matrix, partially demineralized bone matrix, demineralized bone matrix, anorganic bone matrix, deorganified bone matrix, or mixtures thereof.

70. The composition of claim 67 or 68, wherein the peptides or protein fragments are derived from a growth factor.

71. The composition of claim 70, wherein the growth factor is selected from the group consisting osteogenic factors, vascularizing factors, macrophage colony stimulating factor (MCSF), insulin-like growth factors (IGF), angiogenic factors, osteonectin, alpha-2-HS glycoprotein, transforming growth factor (TGF), and bone morphogenic protein (BMP).

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72. The composition of claim 71 wherein the BMP is selected from the group consisting of BMP2, BMP3, BMP5, BMP6, BMP7, BMP8, BMP9, BMP10, BMP11, BMP12, or BMP13.

73. The composition of claim 67 or 68, wherein the peptides or protein fragments are derived from non-collagenous proteins.

74. The composition of claim 67, wherein the inactive protein is a proprotein.

75. The composition of claim 74, wherein the proprotein is a proTGF.

76. The composition of claim 75, wherein the proTGF is proTGF-β.

77. The composition of claim 74, wherein the proprotein is proBMP.

78. The composition of claim 77, wherein the proBMP is proBMP-4 or BMP-2.

79. The composition of claim 74, wherein the protease is a proprotein convertase protease.

80. The composition of claim 67 or 68, wherein the osteoinductive peptides or protein fragments are modified.

81. The composition of claim 67 or 68, wherein the osteoinductive peptides or protein fragments are covalently or non-covalently attached to the matrix.

82. The composition of claim 67 or 68, wherein the bone matrix composition further comprises one or more growth factors having osteogenic activity.

83. The composition of claim 67 or 68, wherein the bone repair matrix further comprises at least one hormone.

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84. The composition of claim 83, wherein the hormone is selected from the group consisting of estrogen, prostaglandin E2, and parathyroid hormone (PTH).

85. The composition of claim 67 or 68, further comprising one or more bioactive agents.

86. The composition of claim 85, wherein the bioactive agent is selected from the group consisting of small molecules, chemical compounds, cells, polynucleotides, proteins, peptides, drugs, and viruses.

87. The composition of claim 85, wherein the bioactive agent is selected from the group consisting of antibiotics, anti-neoplastic agents, growth factors, hematopoietic factors, wound healing factors, and nutrients.

88. The composition of claim 67 or 68, wherein the composition further comprises one or more anti-inflammatory or pro-inflammatory modulators or drugs.

89. The composition of claim 67 or 68, wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 10% or greater.

90. The composition of claim 67 or 68,wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 20% or greater.

91. The composition of claim 67 or 68, wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 35% or greater.

92. The composition of claim 67 or 68, wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 50% or greater.

93. The composition of claim 67 or 68, wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 100% or greater.

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94. The composition of claim 67 or 68, wherein the increase in osteoinductivity of the treated matrix compared to an untreated matrix is at least 200% or greater.

95. A chondrogenic cartilage repair composition for implantation at a cartilage defect site which comprises a cartilage repair matrix treated with at least one protease, wherein the protease causes cleavage of inactive proteins to generate active chondrogenic peptides or protein fragments, wherein the chondrogenic activity of the treated matrix compared to an untreated matrix is increased resulting in improved cartilage formation.

96. A chondrogenic cartilage repair composition for implantation at a cartilage defect site which comprises a cartilage repair matrix treated with a chemical or condition, wherein the chemical or condition generates active chondrogenic peptides or protein fragments, wherein the chondrogenic activity of the treated matrix compared to an untreated matrix is increased resulting in improved cartilage formation.

97. The composition of claim 95 or 96, wherein the chondrogenic peptides or protein fragments are derived from a growth factor.

98. The composition of claim 97, wherein the growth factor is selected from the group consisting osteogenic factors, vascularizing factors, macrophage colony stimulating factor (MCSF), insulin-like growth factors (IGF), angiogenic factors, osteonectin, alpha-2-HS glycoprotein, transforming growth factor (TGF), and bone morphogenic protein (BMP).

99 The method of claim 98, wherein the BMP is selected from the group consisting of BMP2, BMP3, BMP5, BMP6, BMP7, BMP8, BMP9, BMP10, BMP11, BMP12, or BMP13.

100. The method of claim 95 or 96, wherein the peptides or protein fragments are derived from non-collagenous proteins.

101. The composition of claim 95, wherein the inactive protein is a proprotein.

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102. The composition of claim 101, wherein the proprotein is a proTGF.

103. The composition of claim 102, wherein the proTGF is proTGF-β.

104. The composition of claim 101, wherein the proprotein is proBMP.

105. The composition of claim 104, wherein the proBMP is proBMP-4 or proBMP-2.

106. The composition of claim 101, wherein the protease is a proprotein convertase protease.

107. The composition of claim 95 or 96, wherein the peptides or protein fragments are modified.

108. The composition of claim 95 or 96, wherein the chondrogenic peptides or protein fragments are covalently or non-covalently attached to the matrix.

109. The composition of claim 95 or 96, wherein the cartilage repair matrix composition further comprises one or more growth factors having chondrogenic activity.

110. The composition of claim 95 or 96, wherein the cartilage repair matrix composition further comprises one or more hormones.

111. The composition of claim 110, wherein the hormone is selected from the group consisting of estrogen, prostaglandin E2, and parathyroid hormone (PTH).

112. The composition of claim 95 or 96, further comprising one or more bioactive agents.

113. The composition of claim 112, wherein the bioactive agent is selected from the group consisting of small molecules, chemical compounds, cells, polynucleotides, proteins, peptides, drugs, and viruses.

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114. The composition of claim 112, wherein the bioactive agent is selected from the group consisting of antibiotics, anti-neoplastic agents, growth factors, hematopoietic factors, wound healing factors, and nutrients.

115. The composition of claim 95 or 96, wherein the composition further comprises one or more anti-inflammatory or pro-inflammatory modulators or drugs.

116. The composition of claim 95 or 96, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 10% or greater.

117. The composition of claim 95 or 96, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 20% or greater.

118. The composition of claim 95 or 96, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 35% or greater.

119. The composition of claim 95 or 96, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 50% or greater.

120. The composition of claim 95 or 96, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 100% or greater.

121. The composition of claim 95 or 96, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 200% or greater.

122. An implantable bone growth inducing composition comprising:

a bone matrix; and

a peptide or protein fragment that is capable of enhancing the osteoinductivity of the bone matrix resulting in improved bone formation ability as compared to a composition without the peptide or protein fragment.

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123. An implantable cartilage repair graft composition comprising:

a cartilage repair matrix; and

at least one peptide or protein fragment that is capable of enhancing the chondrogenic activity of the cartilage repair matrix resulting in improved cartilage formation ability as compared to a composition without the peptide or protein fragment.

124. The composition of claim 122, wherein the bone matrix comprises mineralized bone matrix, partially demineralized bone matrix, demineralized bone matrix, deorganified bone matrix, anorganic bone matrix, or mixtures thereof.

125. The composition of claim 122 or 123, wherein the peptide or protein fragment is derived from a growth factor.

126. The composition of claim 125, wherein the growth factor is selected from the group consisting osteogenic factors, vascularizing factors, macrophage colony stimulating factor (MCSF), insulin-like growth factors (IGF), angiogenic factors, osteonectin, alpha-2-HS glycoprotein, transforming growth factor (TGF), and bone morphogenic protein (BMP).

127. The composition of claim 126, wherein the BMP is selected from the group consisting of BMP2, BMP3, BMP5, BMP6, BMP7, BMP8, BMP9, BMP10, BMP11, BMP12, or BMP13.

128. The composition of claim 126, wherein the BMP is BMP4 or BMP-2.

129. The composition of claim 126, wherein the TGF is TGF-β.

130. The composition of claim 122 or 123, wherein the peptides or protein fragments are derived from non-collagenous proteins.

131. The composition of claim 122 or 123, wherein the peptide or protein fragment is a hormone or is derived from a hormone.

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132. The composition of claim 122 or 123, wherein the peptide or protein fragment is modified.

133. The composition of claim 122 or 123, wherein the peptides or protein fragments are covalently or non-covalently attached to the matrix.

134. The composition of claim 122, wherein the composition further comprises multiple growth factors having osteogenic activity.

135. The composition of claim 123, wherein the composition further comprises multiple growth factors having chondrogenic activity.

136. The composition of claim 122 or 123, wherein the composition further comprises one or more hormones.

137. The composition of claim 136, wherein the hormone is selected from the group consisting of estrogen, prostaglandin E2, and parathyroid hormone.

138. The composition of claim 122 or 123, further comprising one or more bioactive agents.

139. The composition of claim 138, wherein the bioactive agent is selected from the group consisting of small molecules, chemical compounds, cells, polynucleotides, proteins, peptides, drugs, and viruses.

140. The composition of claim 138, wherein the bioactive agent is selected from the group consisting of antibiotics, anti-neoplastic agents, growth factors, hematopoietic factors, wound healing factors, and nutrients.

141. The composition of claim 122 or 123, wherein the composition further comprises one or more anti-inflammatory or pro-inflammatory modulators or drugs.

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142. The composition of claim 122 or 123, wherein the peptide or protein fragment is purified.

143. The composition of claim 122 or 123, wherein the peptide or protein fragment is not purified.

144. The composition of claim 122 or 123, wherein the increase in chondrogenic or osteoinductive activity of the treated matrix compared to an untreated matrix is at least 10% or greater.

145. The composition of claim 122 or 123, wherein the increase in chondrogenic or osteoinductive activity of the treated matrix compared to an untreated matrix is at least 20% or greater.

146. The composition of claim 122 or 123, wherein the increase in chondrogenic or osteoinductive activity of the treated matrix compared to an untreated matrix is at least 35% or greater.

147. The composition of claim 122 or 123, wherein the increase in chondrogenic or osteoinductive activity of the treated matrix compared to an untreated matrix is at least 50% or greater.

148. The composition of claim 122 or 123, wherein the increase in chondrogenic or osteoinductive activity of the treated matrix compared to an untreated matrix is at least 100% or greater.

149. The composition of claim 122 or 123, wherein the increase in chondrogenic or osteoinductive activity of the treated matrix compared to an untreated matrix is at least 200% or greater.

150. A method of preparing a bone matrix composition, the method comprising the steps of:
providing a bone matrix;

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adsorbing into the bone matrix peptides or protein fragments that are capable of enhancing the osteoinductivity of the bone matrix resulting in improved bone formation ability as compared to a composition without the peptides or protein fragments.

151. A method of preparing a cartilage repair matrix composition, the method comprising the steps of:

providing a cartilage repair matrix;

adsorbing into the matrix peptides or protein fragments that are capable of enhancing the chondrogenic activity of the cartilage repair matrix resulting in improved cartilage formation ability as compared to a composition without the peptides or protein fragments.

152. The method of claim 150 or 151, wherein the peptide or protein fragment is derived from a growth factor.

153. The method of claim 152, wherein the growth factor is selected from the group consisting osteogenic factors, vascularizing factors, macrophage colony stimulating factor (MCSF), insulin-like growth factors (IGF), angiogenic factors, osteonectin, alpha-2-HS glycoprotein, transforming growth factor (TGF), and bone morphogenic protein (BMP).7

154. The method of claim 153, wherein the bone morphogenic protein is selected from the group consisting of BMP2, BMP3, BMP5, BMP6, BMP7, BMP8, BMP9, BMP10, BMP11, BMP12, or BMP13.

155. The method of claim 153, wherein the bone morphogenic protein is BMP4.

156. The method of claim 153, wherein the TGF is TGF-β.

157. The method of claim 150 or 151, wherein the peptides or protein fragments are derived from non-collagenous proteins.

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158. The method of claim 150 or 151, wherein the peptide or protein fragment is a hormone or is derived from a hormone.

159. The method of claim 150 or 151, wherein the hormone is selected from the group consisting of estrogen, prostaglandin E2, and parathyroid hormone (PTH).

160. The method of claim 150 or 151, wherein the peptides or protein fragments are modified.

161. The method of claim 150 or 151, wherein the peptides or protein fragments are covalently or non-covalently attached to the matrix.

162. The method of claim 150, wherein the composition further comprises multiple growth factors having osteogenic activity.

163. The method of claim 151, wherein the composition further comprises multiple growth factors having chondrogenic activity.

164. The method of claim 150 or 151, wherein the composition further comprises one or more hormones.

165. The method of claim 164, wherein the hormone is selected from the group consisting of estrogen, prostaglandin E2, and parathyroid hormone.

166. The method of claim 150 or 151, further comprising one or more bioactive agents.

167. The method of claim 166, wherein the bioactive agent is selected from the group consisting of small molecules, chemical compounds, cells, polynucleotides, proteins, peptides, drugs, and viruses.

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168. The method of claim 166, wherein the bioactive agent is selected from the group consisting of antibiotics, anti-neoplastic agents, growth factors, hematopoietic factors, wound healing factors, and nutrients.

169. The method of claim 150 or 151, wherein the composition further comprises one or more anti-inflammatory or pro-inflammatory modulators or drugs.

170. The method of claim 150 or 151, wherein the peptide or protein fragment is purified.

171. The method of claim 150 or 151, wherein the peptide or protein fragment is not purified.

172. The method of claim 150, wherein the increase in osteoinductive activity of the treated matrix compared to an untreated matrix is at least 10% or greater.

173. The method of claim 150, wherein the increase in osteoinductive activity of the treated matrix compared to an untreated matrix is at least 20% or greater.

174. The method of claim 150, wherein the increase in osteoinductive activity of the treated matrix compared to an untreated matrix is at least 35% or greater.

175. The method of claim 150, wherein the increase in osteoinductive activity of the treated matrix compared to an untreated matrix is at least 50% or greater.

176. The method of claim 150, wherein the increase in osteoinductive activity of the treated matrix compared to an untreated matrix is at least 100% or greater.

177. The method of claim 150, wherein the increase in osteoinductive activity of the treated matrix compared to an untreated matrix is at least 200% or greater.

178. The method of claim 151, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 10% or greater.

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179. The method of claim 151, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 20% or greater.

180. The method of claim 151, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 35% or greater.

181. The method of claim 151, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 50% or greater.

182. The method of claim 151, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 100% or greater.

183. The method of claim 151, wherein the increase in chondrogenic activity of the treated matrix compared to an untreated matrix is at least 200% or greater.

184. A method of treating a bone defect, the method comprising steps of :

implanting the bone matrix composition of claim 65, 66, 92, 93, 118 or 119 into an animal at the site of a bone defect.

185. The method of claim 189, wherein the animal is a human.

186. The method of claim 189, wherein the bone defect is a fracture.

187. The method of claim 189, wherein the bone defect is a congenital bone defect.

188. The method of claim 189, wherein the bone defect is iatrogenic.

189. The method of claim 189, wherein the bone matrix composition has an osteoinductivity score of at least 1 in an animal model or humans.

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190. The method of claim 189, wherein the bone matrix composition has an osteoinductivity score of at least 2 in an animal model or in humans.

191. The method of claim 189, wherein the bone matrix composition has an osteoinductivity score of at least 3 in an animal model or in humans.

192. The method of claim 189, wherein the bone matrix composition has an osteoinductivity score of at least 4 in an animal model or in humans.

193. A bone or cartilage matrix composition comprising:

a bone or cartilage matrix; and

an antisense nucleic acid that decreases expression of BCEF or BCIF mRNA.

194. A bone or cartilage matrix composition comprising:

a bone or cartilage matrix; and

a vector that provides a nucleic acid template for transcription of an antisense nucleic acid that decreases expression of BCEF or BCIF mRNA.

195. A method of increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising the step of introducing an antisense nucleic acid that decreases expression of BCEF or BCIF mRNA or a vector that provides a nucleic acid template for transcription of an antisense nucleic acid that decreases expression of BCEF or BCIF mRNA into the matrix.

196. A bone or cartilage matrix composition comprising:

a bone or cartilage matrix; and

a ribozyme designed to cleave BCEF or BCIF mRNA.

197. A bone or cartilage matrix composition comprising:

a bone or cartilage matrix; and

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a vector that provides a nucleic acid template for transcription of a ribozyme designed to cleave BCEF or BCIF mRNA.

198. A method of increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising the step of introducing a ribozyme designed to cleave BCEF or BCIF mRNA or a vector that provides a nucleic acid template for transcription of a ribozyme designed to cleave BCEF or BCIF mRNA into the matrix.

199. A bone or cartilage matrix composition comprising:

a bone or cartilage matrix; and

an RNAi-mediating agent that decreases expression of BCEF or BCIF mRNA when present within a cell.

200. A bone or cartilage matrix composition comprising:

a bone or cartilage matrix; and

a vector that provides a nucleic acid template for transcription of an RNAi-mediating agent that decreases expression of BCEF or BCIF mRNA when present within a cell.

201. The composition of claim 199 or 200, wherein the RNAi-mediating agent is an siRNA or shRNA.

202. A method of increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising the step of introducing an RNAi-mediating agent that decreases expression of BCEF or BCIF mRNA or a vector that provides a nucleic acid template for transcription of an RNAi-mediating agent that decreases expression of BCEF or BCIF mRNA into the matrix.

203. A bone or cartilage matrix composition comprising:

a bone or cartilage matrix; and

a transcription modulator, wherein the transcription modulator modulates transcription of a BCEF or BCIF.

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204. The composition of claim 203, wherein the transcription modulator is a small molecule, a transcription factor, an engineered transcription modulating protein, or a vector that provides a template for intracellular synthesis of a transcription factor or engineered transcription modulating protein.

205. A method of increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising the step of introducing a transcription modulator into the matrix, wherein the transcription modulator modulates transcription of a BCEF or BCIF.

206. The method of claim 205, wherein the transcription modulator is a small molecule, a transcription factor, an engineered transcription modulating protein, or a vector that provides a template for intracellular synthesis of a transcription factor or engineered transcription modulating protein.

207. A method of increasing the osteoinductive, osteoconductive, or chondrogenic properties of a bone repair matrix or a cartilage repair matrix comprising the step of introducing a cell comprising a modified regulatory sequence into the matrix, wherein the modified regulatory sequence is operably linked to a nucleic acid that provides a template for transcription of a BCEF or BCIF and increases or decreases transcription factor binding affinity or transcription relative to a corresponding naturally occurring regulatory sequence lacking such modification.

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Abstract

The present invention provides methods of improving the osteoinductivity of a bone matrix by activating peptides and protein fragments in the bone matrix that have osteoinductivity. The present invention further provides cartilage repair matrices having increased chondrogenic activity by activating peptides and protein fragments in the cartilage repair matrix that have chondrogenic activity. The present invention further provides improved bone and cartilage matrix compositions having peptides or protein fragments with osteoinductive or chondrogenic activity, respectfully. Methods are provided wherein a protease, chemical, or condition is added to bone or cartilage matrix to generate peptides or protein fragments having osteoinductivity or chondrogenic activity. For example, osteoinductive factors such as TGF, BMP, and IGF are activated by a protease, and the activated factors work to recruit cells from the preivascular space to the site of injury and to cause differentiation into bone- or cartilage-forming cells. Alternatively, the active peptides or protein fragments are added directly to the bone or cartilage matrix. The invention further provides methods of preparing, testing, and using the inventive improved osteodinductive and chondrogenic matrix compositions.

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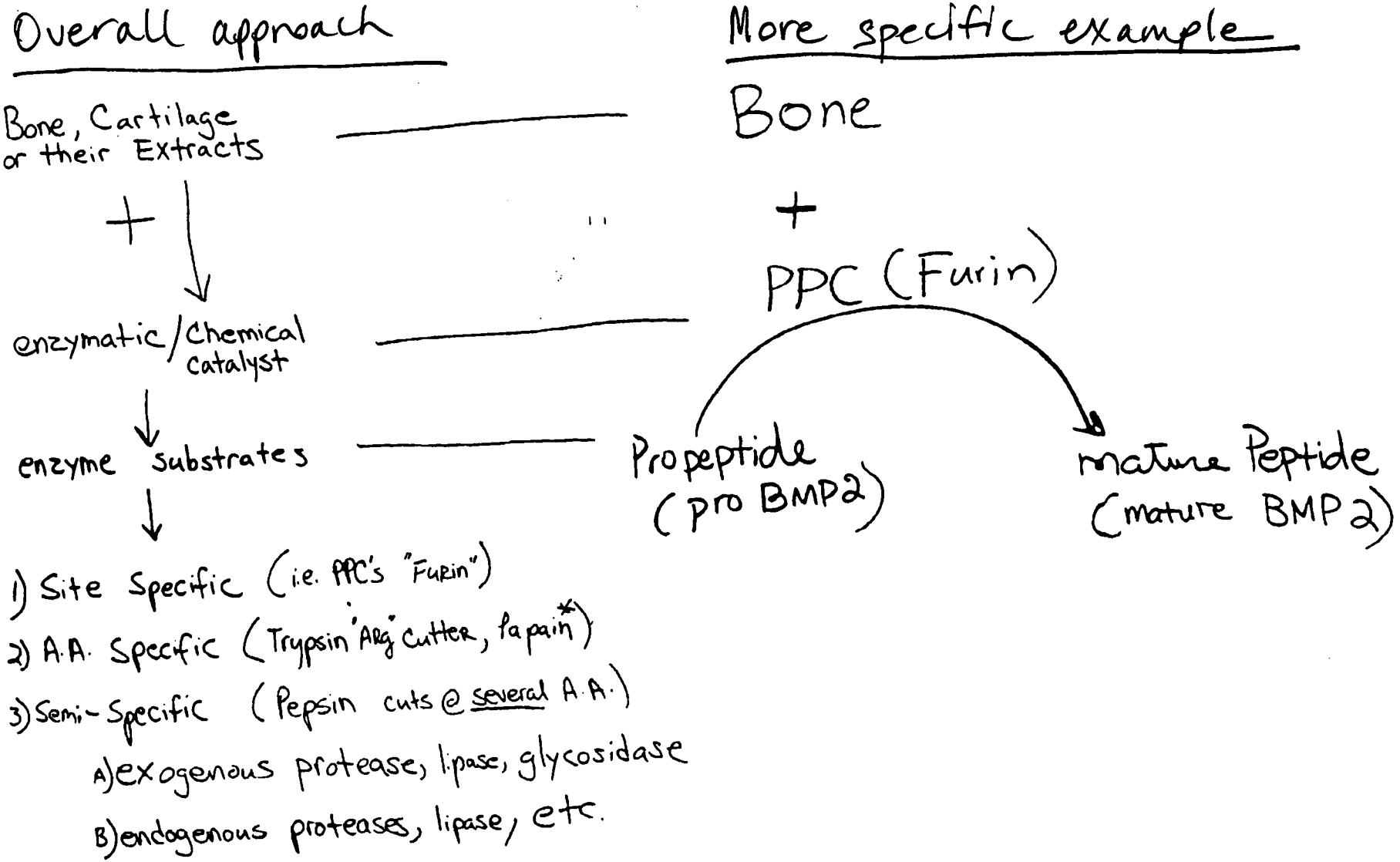
Overall approach
More specific example
Bone, Cartilage or their Extracts
Bone
+
+
PPC (Furin)
enzymatic/Chemical catalyst
enzyme substrates
Propeptide (pro BMP2)
mature Peptide (mature BMP2)
1) Site Specific (i.e. PPC's "Furin")
2) A.A. Specific (Trypsin 'Arg' cutter, Papain*)
3) Semi-Specific (Pepsin cuts @ several A.A.)
A) exogenous protease, lipase, glycosidase
B) endogenous proteases, lipase, etc.

FIGURE 1